



UNIVERSITY
OF TASMANIA

Protein and energy nutrition of brook trout,
***Salvelinus fontinalis* (Mitchill, 1814)**

By

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Declaration

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Abstract

Temperature affects the growth and nutrient utilisation of fish. Diet formulations to the specific protein and energy requirements of brook trout, *Salvelinus fontinalis*, particularly at higher temperatures will be required for sustainable production. The optimum temperature for brook trout growth is 15°C, however, in Australia, summer water temperature is often elevated to about 19°C. Considering this, protein and energy requirements of brook trout were determined at 15°C and 19°C. Using a dose-response model, the digestible dietary protein requirement of brook trout for optimum growth rate was 44% and 40% at 15°C and 19°C, and for optimum protein efficiency was 39% and 35% at 15°C and 19°C, respectively. Using factorial modelling, the maintenance requirements for digestible protein were $0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ (15°C) and $0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ (19°C) and for energy were $29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ (15°C) and $36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ (19°C).

Specific growth rate, feed utilisation indices and apparent digestibility of nutrients were significantly higher at 15°C. Higher levels of gelatinised carbohydrate increased protein efficiency and although liver glycogen storage was increased, it did not cause any pathological symptoms in the liver or intestine. The activity of glycolytic enzyme (PK) in the liver was increased with increasing levels of gelatinised carbohydrate and with higher temperature. Liver lipogenic enzyme (G6PDH) activity was neither affected by temperature nor dietary gelatinised carbohydrate level. Brook trout used gelatinised maize starch effectively for energy at 26%, and at least 13% dietary gelatinised carbohydrate should be added to brook trout feeds to reduce protein catabolism (GDH activity).

The effect of replacing energy from lipid with carbohydrate on growth performance, nutrient utilisation and digestibility of brook trout was evaluated at 15°C and 19°C. Energy source had no effect on growth, protein utilisation and feed utilisation. At both temperatures, 26% carbohydrate improved the apparent digestibility of dry matter (AD_{DM}), gross energy (AD_{GE}) and energy from carbohydrate ($\text{AD}_{\text{CHO-E}}$). Higher levels of gelatinised carbohydrate increased the activity of α -amylase; however, at 19°C activity was lower than at 15°C.

There is great potential for high levels of gelatinised maize starch as an alternative energy source in brook trout diets to replace protein and lipid without compromising the

growth or the function of liver and intestine. While growth was better at 15°C, carbohydrate effectively met the increased energy requirements at 19°C. This study has defined digestible protein and energy requirements for brook trout which can be applied to commercial feeds for production under optimum and challenging summer conditions.

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LIST OF ABBREVIATIONS

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	Serotonin
5-HTP	5-hydroxytryptophan
6PGDH	6-phosphogluconate dehydrogenase
AD _{CHO-E}	Apparent digestibility of energy from carbohydrate
AD _{CP}	Apparent digestibility of protein
AD _{DM}	Apparent digestibility of dry matter
AD _{GE}	Apparent digestibility of gross energy
ALAT	Alanine aminotransferase
ANCOVA	Analysis of co- variance
ANOVA	Analysis of variance
ASAT	Asparate aminotransferase
BO11C	A trade name of pre-gelatinised maize starch
BW	Body weight
CHO	Carbohydrate
CHO:L	Dietary carbohydrate and lipid ratio
CMC	Carboxymethyl cellulose
CP	Crude protein
DE	Digestible energy
DEI	Digestible energy intake
DE _{maint}	Digestible energy requirement for maintenance
DE _{total}	Daily energy requirement (total)
DO	Dissolved oxygen
DP	Digestible protein
DP:DE	Digestible protein and energy ratio
DPI	Digestible protein intake
DP _{maint}	Digestible protein requirement for maintenance
DP _{total}	Daily protein requirement (total)
EG	Eosinophilic granulocytes
FBPase	Fructose-1, 6-bisphosphatase
FCE	Feed conversion efficiency (weight gain·dry feed fed ⁻¹)
FCR	Feed conversion ratio (dry feed fed·weight gain ⁻¹)

FER	Feed efficiency ratio (weight gain·dry feed fed ⁻¹)
FI	Feed intake
G	Gelatinised
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PDH	Glucose 6-phosphate dehydrogenase
GC	Goblet cells
GCHO	Gelatinised carbohydrate
GDH	Gulatamate dehydrogenase
GK	Glucokinase
HK	Hexokinase
HNO ₃	Nitric acid
HSI	Hepatosomatic index
K _{DE}	Partial efficiency of digestible energy
K _{DP}	Partial efficiency of digestible protein
KNO ₃	Potassium nitrate
LNAA	Large neutral amino acids
LP	Lamina propria
MF	Mucosal fold
mRNA	Messenger RNA (Ribonucleic acid)
NADPH	Nicotinamide adenine dinucleotide phosphate
NG	Non-gelatinised
PAS	Periodic acid-schiff
PC	Pyruvate carboxylase
PER	Protein efficiency ratio (weight gain·protein fed ⁻¹)
PEV (%)	Productive energy value
PFK-1	Phosphofrukto kinase
PK	Pyruvate kinase
PLV (%)	Productive lipid value
PPV (%)	Productive protein value
PPV _D (%)	Productive protein value (digestible protein fed basis)
SD	Standard deviation
SDA	Standard dynamic action

SE	Standard error
SGR	Specific growth rate ((%·d ⁻¹)
SM	Submucosa
SNV	Supranuclear vacuolisation
SPSS	Statistical Package for the Social Sciences

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Temperature is an important ecological factor which affects the physiological activity including growth, feed intake, and nutrient utilisation of fish. Commercial culture conditions in Australia often reach the upper thermal limit for brook trout during the summer months. This thesis describes the nutrient utilisation of brook trout, *Salvelinus fontinalis*, (Mitchill, 1814) in relation to temperature. The protein requirement of brook trout in relation to temperature has been determined by dose-response experiment. A quantitative estimation of protein and energy demand of this species for growth and maintenance has also been described by factorial modelling. Based on these experiments and models, a theoretical feed specification chart for this species is presented, which may be effective for brook trout farming.

1.2. Brook trout aquaculture

In last three decades (1980-2010), global aquaculture production has increased by almost 12 times, at an average rate of 8.8% per annum (FAO, 2012). Since the beginning in of 1990s, salmonids contributed more than half of the world production of diadromous fish (FAO, 2012). Salmonids are also one of the most valuable aquaculture species in Australia. The production value of salmonid culture contributed 42% of total aquaculture production value in the financial year 2009-2010 (ABARES, 2011). This production has increased by 13% annually and is predicted to increase further with the rapid expansion of Tasmanian salmon production (ABARES, 2011). Tasmania's salmonid production contributes about 98% of the Australian salmonid production value (ABARES, 2011). Salmonid culture in Tasmania has been mainly concentrated on Atlantic salmon, *Salmo salar* (90%), rainbow trout, *Oncorhynchus mykiss* (9%) and brook trout (1%) in sea cages (Battaglene et al., 2008). Brook trout has shown better growth than rainbow trout in freshwater farming conditions (Okumuş et al., 1999). Although, brook trout showed the highest consumer acceptance over the rainbow trout (Koese et al., 2001), their aquaculture has not been expanded due to technical issue in marine sea cage farming. However, duration of marketable size of brook trout and rainbow trout could be similar when it was cultured fresh water flow-through system (Fischer et al., 2009). Compared to rainbow trout, brook trout have significantly higher protein, lipid and dry matter content in both whole-body and fillet, but have less

slaughter yield (Rasmussen and Ostensfeld, 2000). Brook trout have very high flesh quality with excellent pigmentation, texture and composition and commanding market price.

Brook trout are closely related to arctic char, *Salvelinus alpinus* and are not a true trout (Fischer et al., 2009). They are carnivores and feed on aquatic invertebrates, fish and small vertebrates (Scott and Crossman, 1973). All of the chars are grouped into the genus, *Salvelinus*, are freshwater and anadromous and confined to the Arctic region of the Atlantic and the Pacific oceans and adjacent seaboard (Clements, 1988). Brook trout, *Salvelinus fontinalis* are native to the Atlantic seaboard of North America and are introduced to Australia (Clements, 1988; Jones et al., 1996) and are commercially important coldwater aquaculture species in the United States (Fischer et al., 2009).

Brook trout have been generally cultured for recreational and sport fisheries (Okumuş and Başçınar, 2002). More farming and marketing of this fish could enhance product diversity of salmonid culture (Rasmussen and Ostensfeld, 2000). Brook trout are a hardy salmonid species and can adapt to a variety of culture conditions (Jobling et al., 2010). Their biology has been well studied (Jones et al., 1996; O'Keefe and Benfey, 1999; Haffray et al., 2009), however, optimum conditions for commercial culture, diet utilisation and nutrient requirements have yet to be established (Jobling et al., 2010). Brook trout have more efficient protein retention and feed utilisation than rainbow trout (Rasmussen and Ostensfeld, 2000), however, brook trout are being fed with rainbow trout feeds in commercial culture (Jobling et al., 2010). Although brook trout grow well on rainbow trout feeds, feed formulated to meet their species-specific requirement could enhance flesh quality and culture performance.

Many salmonid producing regions worldwide are experiencing increasing temperatures (Lorentzen, 2008; Ng et al., 2010; Lough and Hobday, 2011). In Australia, cultured salmonids are exposed to temperatures that are toward the upper end of their thermal tolerance range (Battaglene et al., 2008; Pankhurst and King, 2010; Barnes et al., 2011). Higher summer temperatures of 19 to 20°C are routinely encountered on Tasmanian sea cage salmon farms (Fig 1.1; Miller et al., 2006; Ng et al., 2010; Lough and Hobday, 2011), which can increase protein and energy demand, thermal stress and reduce

growth. Consequently, salmon feed formulations need to be adjusted with season (Hemre and Sandnes, 2008). Carbohydrate is more efficient during summer and it is found that winter feeds should contain less carbohydrate and high protein and lipid level as compared to summer, to maximise feed utilisation (Hemre et al., 1995a; Hemre and Sandnes, 2008). Most nutrient requirements of fish species are determined at optimal temperatures. For expanded brook trout production, the key objective of this study is to determine the nutrient utilisation at elevated summer temperature.

1.3. Temperature and growth of brook trout

Brook trout are cold water stenotherms (Lessard and Hayes, 2003) and inhabit water temperatures ranging 0°C to 20°C (Power, 1980). Temperature dependant growth of brook trout has been determined in many studies where the maximum growth of brook trout was observed at 13°C to 14°C (Baldwin, 1957; McMahon et al., 2007; Fischer et al., 2009; Robinson et al., 2010), however, they prefer the temperature range of 11°C to 19°C (Graham, 1949; Clements, 1988). The thermal growth coefficient (TGC) model was used to predict the growth rates of brook trout at a range of temperatures from 5 to 15°C and the highest TGC value was found at 15°C (Gunther et al., 2007).

Fish growth rates increase with increasing water temperature until the maximum growth is reached at an optimal temperature and then decrease as the upper thermal tolerance is reached (Brett, 1979; Jobling, 1997; Katersky and Carter, 2007). With increasing temperatures, fish satisfy their energy requirement by increasing feed intake (Kaushik and Médale, 1994). However, when fish were given an excess supply of food, initially feed intake increased with temperature to a maximum at the optimal temperature (Fig 1.2; Jobling, 1994; Jobling, 1997). The optimum temperature for feed intake is generally a few degrees higher than that of growth; and peaks closer to the upper thermal limit (Fig 1.2; Jobling, 1994; Jobling, 1997). Metabolic energy demand increases exponentially with increasing temperature (Fig. 1.2) and growth efficiency declines due to decreased feed intake and an increase in metabolism (Jobling, 1994; Jobling, 1997; Katersky and Carter, 2005). Thus, as temperature continues to increase above the optimum, growth performance of fish at high temperatures reduce (Person-Le Ruyet et al., 2006; Katersky and Carter, 2007).

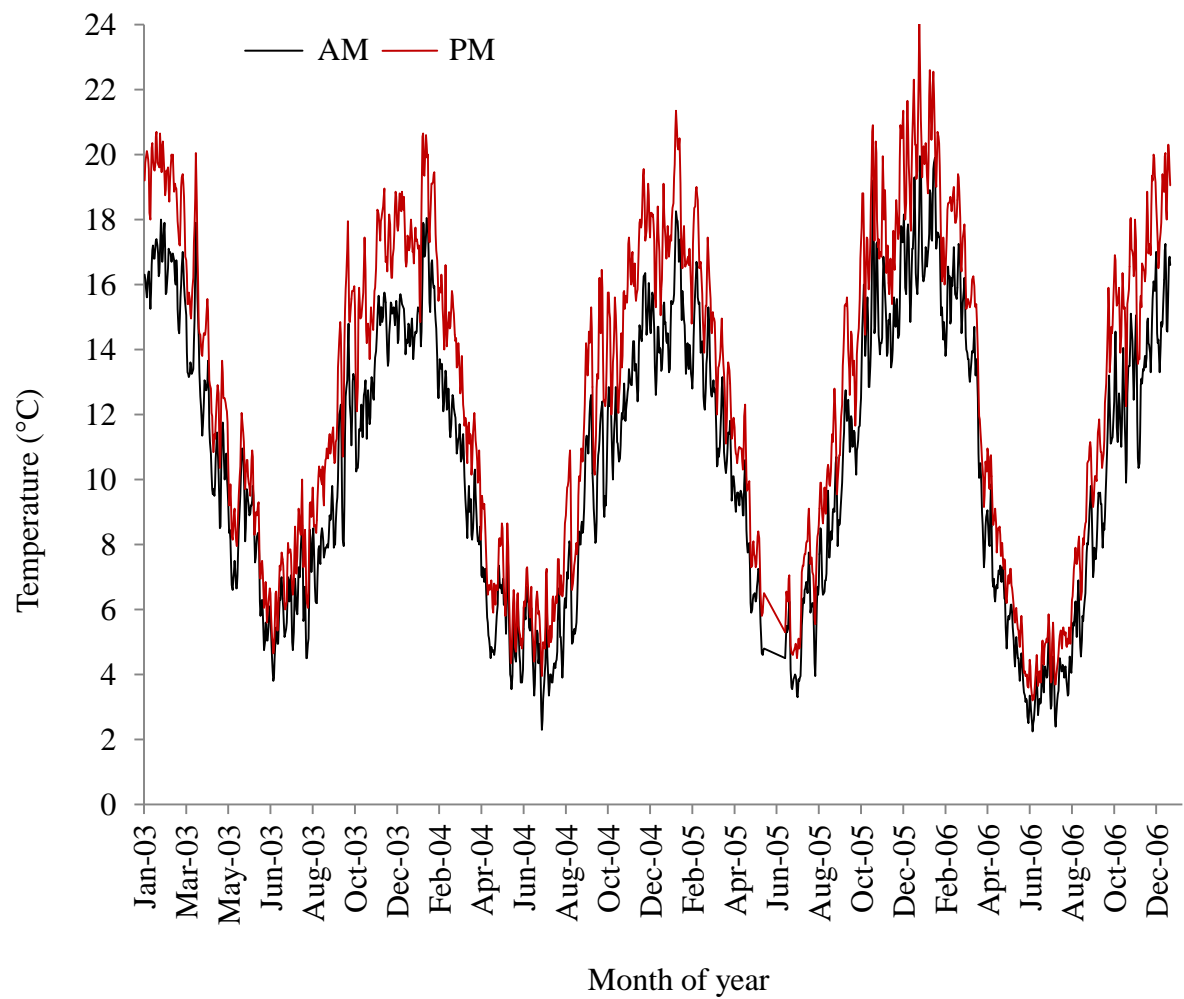


Figure 1.1. Annual water temperatures measured in a Tasmanian brook trout freshwater farm.

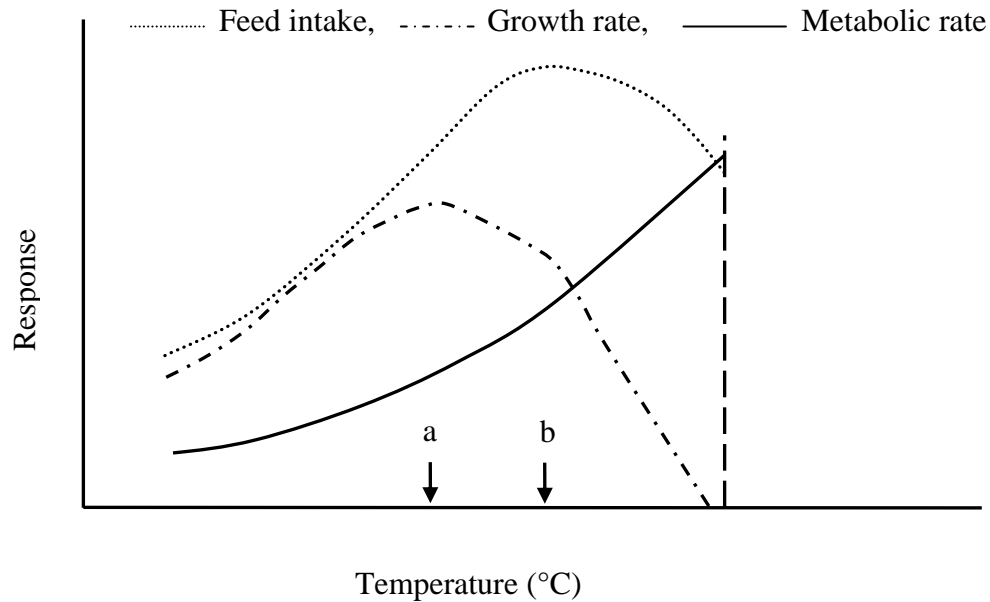


Figure 1.2. Effects of temperature on feed intake, growth rate and metabolic rate of fish. The optimal temperature for growth (a) is slightly lower than the optimal temperature for feed intake (b). The vertical dashed line is the upper thermal tolerance of fish (modified from Jobling, 1994). Both axes are not fitted to scale.

The relationship between feed intake and growth is often termed as growth efficiency or feed conversion efficiency (FCE) which was maximised at temperatures which was near to the optimal temperature for growth (Jobling, 1994; Jobling, 1997; Guerreiro et al., 2012a). At a given ration, the scope for growth rate was reduced progressively at high temperatures due to a marked increase in energy requirement for maintenance (Jobling, 1994; Shearer, 1994). Feed intake of brook trout was higher at 13°C when compared with 9°C and 17°C (Baldwin, 1957). Salmonid growth rates increased with increasing temperature ranging from 4 - 16°C (Austreng et al., 1987), however, growth rate of Atlantic salmon at 19°C was fell about 20% compared to 13°C (Hevrøy et al., 2013). A 50% reduction in feed intake, feed utilization and growth of large Atlantic salmon (2 kg) was observed at 19°C compared to 14°C (Hevrøy et al., 2012). Similarly, reduced feed intake and lower feed utilisation have been found in small Atlantic salmon (<300g) (Handeland et al., 2008) and in barramundi (5g) (Katersky and Carter, 2005) at temperatures higher than the optimum. Considering these backgrounds, it can be clearly stated that aquaculture practices at optimum temperatures are better, however, due to elevated temperature experienced in aquaculture, growth and feed utilization of aquaculture species should be determined at sub-optimum temperature.

In practical farming, high temperatures reduce oxygen concentration in the water and consequently may produce a hypoxic environment. Lower dissolved oxygen was a possible limiting factor in growth rate of fish at high temperatures (Jobling, 1997). Fish generally respond to hypoxia in one of two ways, they are either oxygen conformers (metabolic rate decreases with decreasing dissolved oxygen) or oxygen regulators (metabolic rate are constant over a wide range dissolve oxygen until a critical threshold is reached, P_{crit}) (Barnes et al., 2011). When P_{crit} is reached the oxygen regulators act as oxygen conformers and metabolic rate is dependent on environmental oxygen concentration (Barnes et al., 2011). Salmonids are oxygen conformers and need high levels of dissolved oxygen (Hughes, 1973), however, the Tasmanian population of Atlantic salmon has a relatively high level of hypoxia tolerance (Barnes et al., 2011).

1.4. Protein and energy requirement

Before detailed investigation of the individual amino acid requirements, determination of crude protein requirement in relation to energy provides a broad indication about the levels of protein required to obtain necessary amounts of amino acids (Kim, 1997; Hauler and Carter, 2001; Ward et al., 2003). It is widely accepted that protein is important for growth; however, when the energy is not available for protein synthesis, growth is compromised. Because of increasing demand and the cost of marine resources, a great deal of research has been done on the replacement for fish meal and fish oil (Carter and Hauler, 2000; Ng et al., 2010; Bowyer et al., 2012). In addition, the total energy content of the diet is being replaced with less expensive energy sources such as carbohydrate.

Nutrient requirement studies in fish have traditionally been conducted by empirical or dose-response growth modelling, in which fish were fed diets with graded levels of specific nutrient and the optimum nutritional requirement was calculated at level which supported the best growth (Shearer, 1995; Lupatsch, 2009). Although the traditional dose-response model is often criticised for limited application to explain requirement under different conditions (Lupatsch, 2009), it provides important information to standardize the inclusion level of nutrients such as protein, lipid and carbohydrate. Requirements based on the dose-response model are also important to further investigate finding alternative protein or energy sources to achieve effective growth.

Protein and energy requirement of fish are often expressed in term of intake (grams or kilojoules of nutrient required per kilogram body weight per day) rather than in percentages (Tacon and Cowey, 1985). Recently, it has been suggested that protein and energy requirements should be expressed on metabolic weight basis (Lupatsch, 2009). The quantitative protein and energy requirement should also be estimated on the basis of the nutrient needed for both nutrient deposition and loss. The losses can be defined the obligatory loss of nutrients such as for metabolic process, locomotion and for aging (i.e., maintenance). The estimation of energy partitioning for the maintenance requirement and growth is necessary to improve the nutrient requirement model, which is termed as factorial approach model (Bureau et al., 2006; Lupatsch, 2009).

Factorial models calculate the requirements on an empirical basis (Lupatsch, 2009). Total energy and protein requirements are calculated as the sum of the relative amounts of protein and energy used to meet basic maintenance functions (Bureau et al., 2006; Lupatsch, 2009). The protein and energy required for growth can be calculated to predict nutritional requirements under different culture conditions, temperatures and fish size (Glencross, 2008; Pirozzi et al., 2010). Since large fish contain more protein and energy than small fish in term of per unit of biomass, the recovered protein and energy can serve to determine the retention efficiency by developing equations to describe the whole-body composition across the life cycle (Dumas et al., 2010). Using factorial models, protein and energy requirements have been determined across the size range of fish required for commercial production for range of species (Glencross, 2008; Lupatsch, 2009; Booth et al., 2010; Van Trung et al., 2011; Amrkolaie et al., 2012) but a few studies have done for salmonids (Helland et al., 2010).

The dose-response requirement model can be used to determine the required balance of nutrients by blending or replacing ingredients to achieve the desired response in growth and nutrient efficiency. However, this model is not suitable to determine the minimum energy requirement for growth, but it is effective to assess the cofounding effect of energy and nutrient ingredients (protein, carbohydrate and lipid). Factorial modelling is effective to determine the minimum requirement of energy as well as energy required for growth above the maintenance. Factorial modelling also calculates requirements on the temperature, size, predicted growth and nutrient deposition. It also allows farmer to control growth by knowing maintenance requirement for different sizes of fish and conditions and predict production. In this, both of these modelling approaches were undertaken to determine nutritional requirements of brook trout.

1.5. Digestibility of maize in fish feed

Maize has been frequently used in fish feed by small scale farmers (Dongmeza et al., 2010) and as a source of energy in salmonid aquafeeds (Pfeffer et al., 1991; Bergot, 1993; Hemre et al., 1996; Sanden et al., 2006). Digestible energy from any complex carbohydrate may be affected by various factors such as botanical origin, physical state and inclusion level (Bergot, 1993; Stone, 2003). Apparent digestibility coefficient

(ADC) of three varieties of maize was compared in rainbow trout diet and found that the ADC of ordinary maize was intermediate (33%) between that of amylomaize (18%) and waxy maize (54%) (Bergot, 1993). Normal maize contains 28-30% of amylose and 70-72% of amylopectin, amylomaize contains 65% amylose and waxy maize contains 1% amylose and 99% amylopectin (Pfeffer et al., 1991; Bergot, 1993). High proportions of amylose have a negative effect on carbohydrate digestibility in fish (Bergot, 1993). Rainbow trout showed better growth and improved energy efficiency when fed waxy maize than normal maize (Pfeffer et al., 1991).

Processing (gelatinisation during extrusion) improves the digestibility of carbohydrate (Bergot, 1993; Stone, 2003; Krogdahl et al., 2005). Improved digestibility in rainbow trout was found with increasing the degree of extrusion of maize (Pfeffer et al., 1991). Apparent digestibility of treated carbohydrate, including maize reached to 95% while digestibility of native treated carbohydrate remained below 60% in the diet of rainbow trout (Bergot, 1993). Gelatinised maize starch in the diet of channel catfish was 17% more digestible than native maize starch (Lovell, 1989b). Generally, maize and wheat starches were more digestible than potato starches (Stone, 2003).

Dietary inclusion levels of maize affect the growth and nutrient efficiency of fish (Wu et al., 2007; Ye et al., 2009). Juvenile yellowfin seabream, *Sparus latus* fed 48% crude protein with four different levels of maize starch (5%, 10%, 20% and 26%) had higher growth when fed 10% or 20% maize starch (Wu et al., 2007). When fish were fed 20% maize it was determined that the highest feed efficiency ratio, protein efficiency ratio and protein productive value occurred (Wu et al., 2007). Juvenile yellow catfish, *Pelteobagrus fulvidraco* fed diets containing 36% protein and 24-36% gelatinised maize starch have been reported for optimal growth and feed utilisation (Ye et al., 2009). However, effect of inclusion levels of maize on growth and nutrient efficiency of salmonids is not available.

1.6. Carbohydrate and feed intake

Dietary carbohydrate affects the feed intake of fish. When dietary fat intake is reduced, the dietary carbohydrate content typically rises in the feeds (Parks and Hellerstein, 2000; Parks, 2001). Higher plasma glucose is generally observed after feeding a

carbohydrate-rich diet (Cowey et al., 1977; Hemre et al., 1989; Wilson, 1994; Brauge et al., 1995; Vielma et al., 2003; Kirchner et al., 2008; Kumar et al., 2008) and high blood glucose suppresses feed intake in Nile tilapia (Tran-Duy et al., 2008). Increasing the content of carbohydrate in the diet also increases the dietary volume i.e. decreases the dietary energy density (Tran-Duy et al., 2008). Fish adjust their dietary energy intake to the digestible energy levels in the diet (Kaushik and Médale, 1994; Morales et al., 1994) and energy intake increases with the increasing level of carbohydrate in the feed (Hemre et al., 1989). As the energy content of high-carbohydrate diets is lower than that of low-carbohydrate diets with high protein and lipid, fish fed high-carbohydrate diets try to compensate for the low dietary energy contents by increasing feed intake, which leads to increase stomach volume (Tran-Duy et al., 2008). Thus, feed intake is generally restricted prior to the energy requirement being fulfilled on account of stomach fullness (Tran-Duy et al., 2008). However, feed intake increased with the increasing level of dietary gelatinised carbohydrate (23%) in Atlantic salmon due to decreased digestibility of gelatinised carbohydrate (Aksnes, 1995).

Feed intake and gastric evacuation rate are closely related and which is irrespective to the carbohydrate level in the diet (Tekinay and Daves, 2002). Gross energy content of the feed is also important determinant of gastric evacuation in the fish (Jobling, 1981; Jobling, 1987). Rainbow trout as a visual feeder is likely to feed for stomach fullness in the short term regardless of the dietary composition (Tekinay and Daves, 2002). However, gastric evacuation rate of lower carbohydrate diet (14.9%) was significantly different than medium (31.4%) and high carbohydrate diet (42.3%) (Tekinay and Daves, 2002).

1.7. Carbohydrate inclusion level

Inclusion level of carbohydrate is different among the species (Table 1.1). Optimum dietary carbohydrate levels recommended for carnivorous fish showed a wide range from 9% for Atlantic salmon to 50% for hybrid *Clarias* catfish (Table 1.1). In term of fillet colour, feed efficiency and dry matter (DM) digestibility, the optimum level of carbohydrate in the diet for Atlantic salmon, *Salmo salar* was approximately 10% (Aksnes, 1995). A carbohydrate inclusion above 9% reduced the digestibility of carbohydrate in the diet (Hemre et al., 1995d). According to Phillips et al. (1948) trout

diets should contain less than 12% digestible carbohydrate and higher level of carbohydrate increased mortality (cited by Bergot, 1979). The tolerable range of carbohydrate in salmonid feeds was recommended from 14 to 20% (Pieper and Pfeffer, 1980; Hilton and Atkinson, 1982; Spannhof and Plantikow, 1983). In contrast, rainbow trout can utilise up to 25% of digestible carbohydrate in diet (Brauge et al., 1994), although 20% digestible carbohydrate showed best growth and FCR of this salmonid fish with constant water temperature of 17.5°C (Kim and Kaushik, 1992). A diet containing 18-27% of gelatinised carbohydrate showed higher feed efficiency in rainbow trout (Yamamoto et al., 2001). High-carbohydrate levels (22% - 37% expanded wheat) showed good rates of growth of rainbow trout (Banos et al., 1998). Currently the commercial trout aquafeeds contain 15.5 to 17.5% carbohydrate (personal communication from Skretting). Since salmonids can utilise carbohydrate in some levels, there is a great interest in using carbohydrate in their feeds to reduce feed cost. Because of this commercial interest in brook trout, it is important to investigate the maximum tolerable limit of carbohydrate if the inclusion level can be increased by using gelatinised maize starch. The important question whether charr can use more gelatinised carbohydrate than salmonids.

Table 1.1. Carbohydrate inclusion level in the diet of fish

Fish species	Weight (g)	Temperature (°C)	Range of carbohydrate (%)	Optimum level (%)	Reference
Group: Omnivorous					
Nile tilapia, <i>Oreochromis niloticus</i>	50-53	28	10-36		(Tran-Duy et al., 2008)
Nile tilapia, <i>Oreochromis niloticus</i>	8.7±2.0	27±0.2	5-35		(Gaye-Siessegger et al., 2006)
Tilapia, <i>Oreochromis niloticus</i> x <i>O. aureus</i>	9.1±0.1	26.6±1.8	6-46	22	(Wang et al., 2005)
Silver barb, <i>Puntius gonionotus</i>	0.59±0.01	-	22-38	26	(Mohanta et al., 2009)
Giant gouramy <i>Osphronemus gouramy</i>	29-32		20.8 - 57	20.8	(Mokoginta et al., 2004)
	Sub adults		21.2-47.5	47.5	
<i>Oreochromis mossambicus</i>	-	-	5-25	20	(Sornaraj and Singh, 2004)
<i>Cyprinus carpio</i>	-	-	5-25	20	(Sornaraj and Singh, 2004)
Group : Herbivorous					
Gibel carp, <i>Carassius auratus</i> var. <i>gibelio</i>)	8.5	19-25	24-40	24-28	(Tan et al., 2009)
Grass carp, <i>Ctenopharyngodon idella</i>	2.27	28-30	17.7-40.5	27.47	(Gao et al., 2010)
Group: Carnivorous					
European sea bass, <i>Dicentrarchus labrax</i>	15	18-25	13-31		(Moreira et al., 2008)
	2.78	-	20-35	<30	(Perez et al., 1997)
Atlantic salmon, <i>Salmo salar</i>	80	-	0-31	9	(Hemre et al., 1995c)
Spotted snakehead, <i>Channa punctatus</i>	-	-	5-25	10	(Sornaraj and Singh, 2004)
Striped snakehead, <i>C. striatus</i>	-	-	5-25	10	(Sornaraj and Singh, 2004)
Gilthead sea bream, <i>Sparus aurata</i>	2.5	21±0.2	5-26	<20	(Fernández et al., 2007)
Hybrid <i>Clarias</i> catfish	22.0±1.0	-	30-60	37-50	(Jantrarotai et al., 1994)
Yellow catfish, <i>Pelteobagrus fulvidraco</i>	8.24±0.20	23.5-28	24-36	24-36 with 36% protein	(Ye et al., 2009)
Largemouth Bass, <i>Micropterus salmoides</i>	128.5±21.5	22.2±0.4	13-25	<20	(Amoah et al., 2008)
Australian snapper, <i>Pagrus auratus</i>	110 and 375	20.6-25.7	15-45	<25% for small group and <35% for large group	(Booth et al., 2006)
African catfish, <i>Clarias gariepinus</i>	12.32±0.04	28±1	15-38	27-38	(Ali and Jauncey, 2004)
Striped bass, <i>Morone saxatilis</i>	-	-	0-25	15-20	(Small and Soares, 1999)
Rockfish, <i>Sebastes schlegeli</i>	3.6±0.2	20±1.07	8.3-33.4	19.8	(Lee and Kim, 2009)
Stinging catfish, <i>Heteropneustes fossilis</i>	-	-	0-30	20	(Erfanullah and Jafri, 1998)
Southern catfish, <i>Silurus meridionalis</i>	-	-	0-30	12-18	(Fu and Xie, 2005)
Pikeperch, <i>Sander lucioperca</i>		23	10-20	15	(Nyina-Wamwiza et al., 2005)

1.8. Carbohydrate as an alternative energy

Carbohydrate is the least expensive source of energy in aquafeeds and is a useful technical ingredient aiding in pellet binding (Hemre et al., 1996). Dietary carbohydrate is not an essential nutrient for carnivorous fish, however, it is catabolised to provide energy and reduces the use of protein and lipid for the synthesis of metabolic intermediates and other biologically important compounds (Wilson, 1994). Higher dietary carbohydrate levels accelerate the whole-body lipid deposition and show a protein sparing effect (Keshavanath et al., 2002). The protein sparing effect of carbohydrate has been established in large rainbow trout (Beamish and Medland, 1986), Atlantic salmon (Hemre et al., 1995b; Grisdale-Helland and Helland, 1997) and sea bass (Hidalgo and Alliot, 1988) indicating that dietary carbohydrate should be an effective energy source for brook trout.

The capacity to utilise carbohydrate by fish varies widely between species due to the anatomy of the digestive tracts, feeding habits and activity of endogenous digestive enzymes (Stone, 2003). Warm water fish are more capable of utilizing higher levels of dietary carbohydrate than coldwater and marine fish (Wilson, 1994). In rainbow trout, carbohydrate digestibility and utilisation as energy were found to be temperature dependent and improved as water temperature increased from 8°C to 18°C (Medale et al., 1991). Carbohydrate is mainly digested in the anterior segment of alimentary tract and low temperatures reduce the solubility of digestive fluid resulting in lower digestibility of carbohydrate (Lovell, 1989a; Lee and Pham, 2011). High levels of carbohydrate increase the volume of intestinal juices and accelerate the passage of chyme through the intestine almost twice as quickly as protein-rich diets and reduced the availability of time for absorption (Spannhof and Plantikow, 1983). Increased non-starch polysaccharide (NSPs) in the diet increases the digesta viscosity in the proximal and distal intestine, reduces the bile acid concentration in digesta leading to lower protein and lipid digestibility (Vissia and Beyen, 2000; Sinha et al., 2011).

The utilisation of carbohydrate is also affected by dietary carbohydrate and lipid levels and is also correlated to temperature. Utilisation of carbohydrate was not affected by carbohydrate and lipid level at 8°C, however, at 18°C it seemed to be higher at lower

carbohydrate level in diet (Brauge et al., 1995). It has been shown in gilthead sea bream, *Sparus aurata* and European sea bass, *Dicentrarchus labrax* that feeding excessive carbohydrate levels at low temperatures exhibited low carbohydrate assimilation due to low digestive enzyme activity, while coldwater species (Atlantic salmon) can adapt their enzymatic digestion at low temperatures and increased carbohydrate utilisation (Papoutsoglou and Lyndon, 2005). However, our understanding of the interaction between dietary carbohydrate, lipid and temperature on carbohydrate utilisation in fish is limited (Guerreiro et al., 2012a; Guerreiro et al., 2012b). Therefore, the dietary carbohydrate and lipid level for brook trout in relation to temperature need to be optimised in order to promote effective utilisation of energy sources.

1.9. Carbohydrate metabolism in fish

Carbohydrate metabolism in fish occurs in the hepatocytes which play an important role to store or produce glucose depending on the need (Klover and Mooney, 2004; Enes et al., 2009). Carbohydrates are absorbed in fish blood as glucose and catabolised to energy through glycolysis and the Krebs cycle under aerobic condition (Fig. 1.3). Excess glucose is either converted to glycogen in the liver through glycogenesis or converted to lipid through lipogenesis (Fig. 1.3). During starvation, the glucose requirement for metabolic purposes may be satisfied by the degradation of glycogen into glucose via glycogenolysis or by *de novo* synthesis, gluconeogenesis (Fig. 1.3).

The common pathway to catabolise glucose in all organisms is called glycolysis which is the oxidation of glucose to pyruvate. There are three essentially irreversible steps in glycolysis (Fig. 1.3): (1) glucose is converted to glucose 6-phosphate by the action of hexokinase (HK) or glucokinase (GK, isomer of hexokinase) (2) fructose 6-phosphate is converted to fructose 1,6-biphosphate by the action of phosphofructokinase (PFK-1) and (3) phosphoenol pyruvate is converted to pyruvate by the action of pyruvate kinase (PK). The regulation of HK or GK with dietary carbohydrate indicates that carbohydrate may be used in subsequent metabolic pathways (glycogenesis and lipogenesis) (Enes et al., 2009). Both temperature and carbohydrate levels can affect the activity of these enzymes. Hexokinase activity was increased at higher temperatures in European sea bass (Moreira et al., 2008). Increasing water temperature enhanced GK and PK

activities in the liver of gilthead sea bream (Couto et al., 2008). Rainbow trout fed 24% glucose diets responded by expressing GK in liver indicating that this fish can utilise the carbohydrate (Panserat et al., 2001b). The level of gelatinised carbohydrate in the diet also affects GK activity (Fig. 1.4). It is commonly believed that GK activity of carnivores is not induced by carbohydrate; however, recent studies showed that high levels of dietary carbohydrate increased GK activity in fish liver (Fig.1.4). Glucokinase activity increased with the increasing dietary gelatinised carbohydrate level from 10 to 20% and reduced with increasing gelatinised carbohydrate level from 20 to 30%, suggesting that 20% dietary carbohydrate is near the maximum tolerable level for metabolic utilisation of carbohydrates by European sea bass juveniles (Moreira et al., 2008).

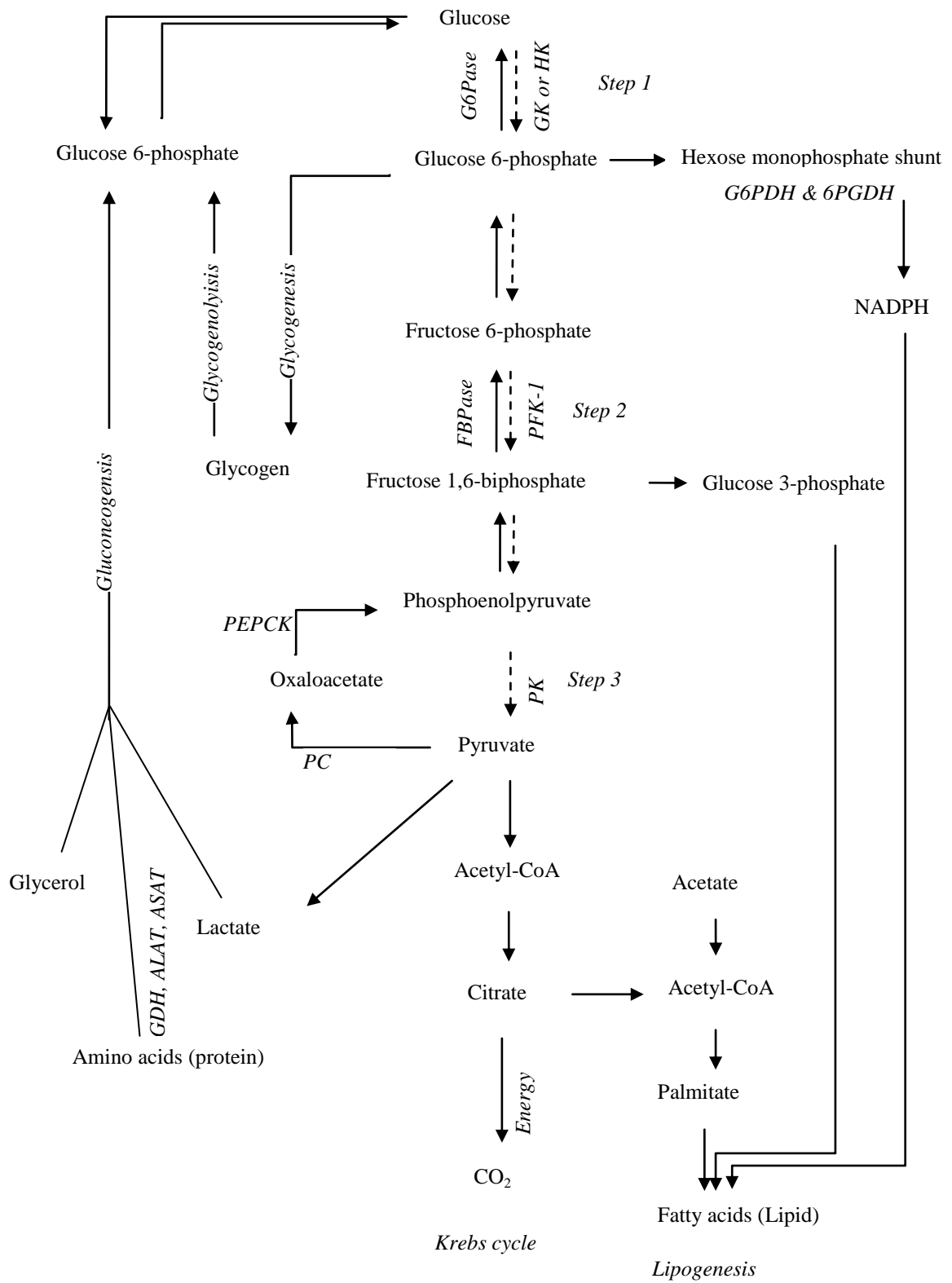
Fish with high PK activities have a high capacity of glycolysis and carbohydrate utilisation (Borrebaek and Christophersen, 2000). Regulation of PK is affected by dietary carbohydrate and it has been found that high level dietary carbohydrates increased the PK activity in carnivores (Fig. 1.5). In gilthead sea bream high dietary carbohydrate improved the hepatic PK activity (Fernández et al., 2007; Couto et al., 2008). However, water temperature affects the hepatic enzymatic activity. Increasing water temperature enhanced liver glucokinase (GK) and pyruvate kinase (PK) activities, suggesting that gilthead sea bream utilise more dietary carbohydrate at higher temperatures (Couto et al., 2008).

When dietary carbohydrate is insufficient to meet requirements, glucose is synthesised from non-carbohydrate precursors such as pyruvate, amino acids, lactate, glycerol, or α -ketoacids by the process of gluconeogenesis. The three essential steps of gluconeogenesis are: (1) pyruvate is converted to oxaloacetate catalysed by pyruvate carboxylase (PC) and then converted to phosphoenolpyruvate using enzyme phosphoenolpyruvate carboxykinase (PEPCK), (2) fructose 1,6-biphospahte is converted to fructose 6-phosphate using fructose 1,6-biphosphatase (FBPase) and (3) glucose 6-phosphate is converted to glucose using glucose 6-phosphatase (G6Pase). In glycolysis, glucose is converted to pyruvate and in gluconeogenesis pyruvate is converted to glucose. Phosphoenolpyruvate carboxykinase is a rate-limiting enzyme in

hepatic gluconeogenesis and plays a central role in glucose homeostasis (Panserat et al., 2001a).

The regulation of gluconeogenesis also depends on two enzymes, fructose 1,6-biphosphatase (FBPase), and glucose 6-phosphatase (G6Pase) (Enes et al., 2009). The increasing activity of these gluconeogenic enzymes, such as PEPCK, FBPase, and G6Pase reflect the dietary requirement of carbohydrate to provide available glucose to meet metabolic needs. In rainbow trout, gilthead seabream, European sea bass and Atlantic salmon, no regulation of PEPCK, FBPase, and G6Pase was found when fish were fed dietary carbohydrate (Enes et al., 2009). This suggest that there is a persistence of high levels of endogenous glucose production in the fish liver due to the absence of gluconeogenesis by dietary carbohydrates (Enes et al., 2009). The higher activity of these FBPase and G6Pase enzymes was observed in fish fed non gelatinised (NG) carbohydrate than gelatinised (G) carbohydrate fed group, suggesting a metabolic demand of glucose in the NG fed group (Kumar et al., 2009). Topmouth culter, *Erythroculter ilishaeformis* fed with a carbohydrate free diet reported high expression levels of PEPCK, consistent with increasing gluconeogenesis (Yu et al., 2007).

Figure 1.3. Metabolic pathway of carbohydrate metabolism in fish. Three essential steps of glycolysis (dash line) by which glucose is converted to pyruvate catalysed by HK, PFK-1 and PK, respectively. Pyruvate is subsequently used in either the Krebs cycle to provide energy or lipogenesis pathway. In case of gluconeogenesis these three steps are bypassed to form glucose, catalysed by pyruvate carboxylase (PC), PEPCK, FBPase and G6Pase, respectively. Another gluconeogenesis pathway is also possible in which glucose is produced from glycerol, fatty acids, lactate and amino acids (action of GDH) to meet glucose requirement. Excess glucose can be converted to lipid through hexosemonophosphate shunt (catalysed by G6PDH and 6GPDH) or to glycogen (modified from Hemre et al., 2002; Rawles et al., 2008; Enes et al., 2009).



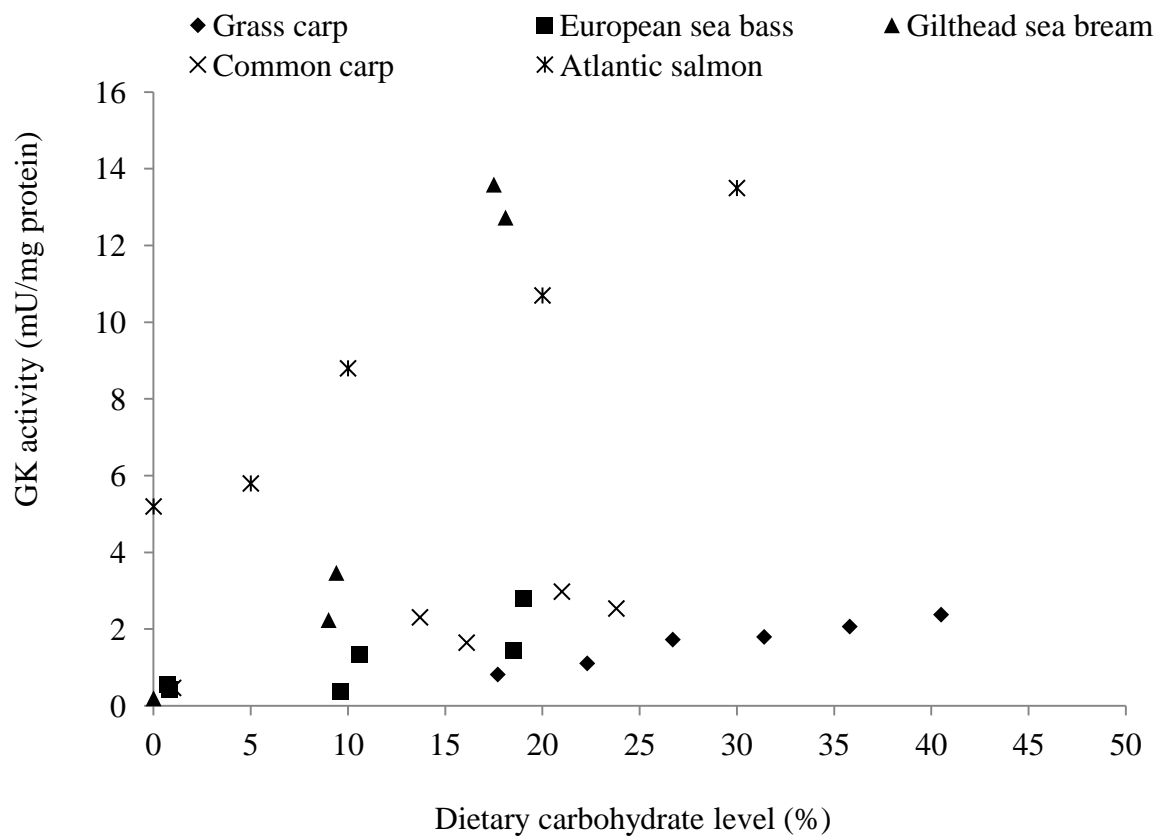


Figure 1.4. Effect of dietary carbohydrate level on the glucokinase activity in the liver of fish (Borrebaek et al., 1993; Capilla et al., 2004; Enes et al., 2006; Enes et al., 2008b; Gao et al., 2010).

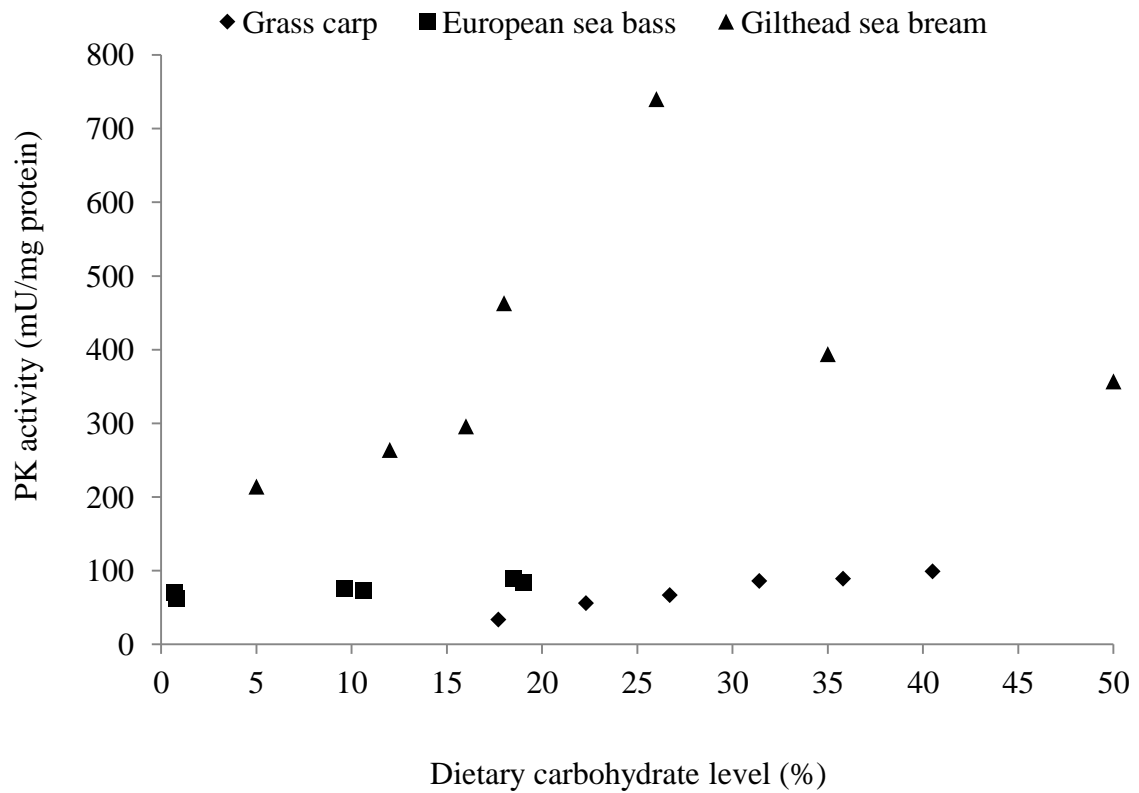


Figure 1.5. Effect of dietary carbohydrate level on the pyruvate kinase activity in the liver of fish (Bonamusa et al., 1992; Fernández et al., 2007; Gao et al., 2010).

Fish fed insufficient dietary carbohydrate may also meet their glucose requirement by catabolising amino acids through *de novo* synthesis of glucose (gluconeogenesis) (Fig. 1.3), catalysed by glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT) or aspartate aminotransferase (ASAT) (Fig. 1.3). Fish fed dietary carbohydrates (normal or waxy maize starch) significantly improved protein utilisation associated with increased glycolytic enzyme activities (GK and PK), as well as decreased gluconeogenic (FBPase) and amino acid catabolic (GDH) enzyme activities (Enes et al., 2006). Increasing water temperature from 18°C to 25°C enhanced the GK but not G6Pase activities European sea bass and gilthead sea bream indicating that fish can utilise glucose from dietary carbohydrate at higher temperature (Enes et al., 2008a).

In fish, excess carbohydrate is possibly converted to lipid (lipidogenesis) through the hexose monophosphate shunt pathway (HMP shunt), also called pentose phosphate pathway (Fig. 1.3). It is the most important alternative pathway, catalysed by action of glucose 6-phosphate dehydrogenase (G6PDH), lactonase, 6-phosphogluconate dehydrogenase (6PGDH) and phosphopentose isomerase. The activities of G6PDH was higher in juvenile spotted Babylon, *Babylonia areolata* fed 20% dietary carbohydrate level (Zhang et al., 2009). In fish, higher values of PFK-1/FBPase and PK/FBPase as well as the increasing activity of G6PDH and 6PGDH indicated that glycolysis and the pentose phosphate pathway was active to metabolise the excess glucose to pyruvate and therefore, enhanced the subsequent lipidogenesis (Metón et al., 2003). The rate of flux through this pathway is correlated positively with feed intake and dietary carbohydrate; and lipid or fatty acid synthesis is increased with carbohydrate (Médale et al., 1999; Hemre et al., 2002). It could be possible to enhance the HMP shunt in fish fed low lipid with high carbohydrate to generate NADPH required for fatty acid synthesis (Hilton and Atkinson, 1982). In contrast, in Nile tilapia, *Oreochromis niloticus*, fed isoenergetic diets containing high or low lipid balanced by carbohydrate showed that whole-body lipid was decreased with high carbohydrate level and *de novo* lipogenesis from carbohydrate was limited (reviewed by Hemre et al., 2002). Similarly in rainbow trout, whole-body lipid decreased with increasing carbohydrate, although G6PDH activity was increased and suggested that some factors might limit the effectiveness of lipogenesis from carbohydrate (Hilton and Atkinson, 1982). There may be an optimum

level of carbohydrate and lipid in trout diets which would maximise the metabolism (Hilton and Atkinson, 1982).

Acetyl-CoA derived from dietary carbohydrate or protein catabolism may also be used *de novo* lipid biosynthesis (Fig. 1.3). Generally high carbohydrate diets tend to lead to the accumulation of whole-body lipid in fish through increased efficiency of lipid biosynthesis from dietary lipid and decreased contribution of dietary lipid to oxidative metabolism (Hemre et al., 2002). The species specific optimum requirements for carbohydrate need to be confirmed to prevent depletion of Krebs cycle intermediates and reduce protein catabolism to fulfil the energy requirement.

1.10. Carbohydrate and protein deposition

Carbohydrates sources may affect the protein deposition in the whole-body of fishes. Protein deposition in tilapia fed with starch was higher than the fish fed with glucose diet (Shiau and Chen, 1993; Lin and Shiau, 1995; Shiau and Liang, 1995). Fish fed with glucose reported the lower whole-body crude protein level compared to those fed with maize grain and starch diet (Alasgah and Ali, 1994). Stinging cat fish, *Heteropneustes fossilis* fed diets containing different sources of carbohydrates (i.e., glucose, fructose, maltose, sucrose, dextrin, pre-cooked corn-starch or alpha-cellulose) at 20% inclusion has been studied and the maximum protein retention rate was reported in the fish fed the dextrin diet (Erfanullah and Jafri, 1999). An experiment involving spotted Babylon fed diet containing 5-30% carbohydrate was conducted and the protein content of the soft tissue increased with increasing dietary carbohydrate level (Zhang et al., 2009). On the hand, the whole body protein content was higher when the diet of silver barb contained 26-34% carbohydrate (Mohanta et al., 2009). High carbohydrate diet did not negatively affect protein synthesis, although protein accretion decreased, suggesting an increase in protein degradation (Viaplana-Marin et al., 2006).

Dietary carbohydrate improved the protein utilisation in spotted babylon, *Babylonia areolata* (Zhang et al., 2009), gilthead sea bream (Enes et al., 2008b). The highest protein efficiency ratio was 3 ± 0.01 in spotted babylon fed diet 20% carbohydrate and this ratio was 1.04 ± 0.06 in gilthead sea bream fed diet containing 17.5% carbohydrate. The

most favourable utilisation of the dietary protein by European eel, *Anguilla anguilla* was exhibited after feeding with the diet containing the highest carbohydrate level with the lowest in protein (Hidalgo et al., 1993). On the other hand, inclusion of carbohydrate in the diet of plaice, *Pleuronectes platessa* showed negative effect on digestion and absorption of protein (Jobling, 1981). Higher protein efficiency ratios was found in rainbow trout fed diet containing 18-27% carbohydrate (Yamamoto et al., 2001).

1.11. Aims of the study

Although salmonid aquaculture is facing increasing summer temperatures, there is limited information on the interaction between temperature and nutrition in brook trout. Information on the optimum protein-to-energy ratio required for brook trout in relation to temperature is not available. In addition, there is a lack of literature about the optimum balance of macronutrients (protein, lipid and carbohydrate) in brook trout aquafeeds. Like other carnivorous fish, brook trout may be able to utilise gelatinised carbohydrate, however, optimum balance of carbohydrate with other macronutrients (protein and lipid) needs to be determined for better growth and nutrient efficiency. Therefore several experiments have been conducted to address the following aims:

Chapter 2

- To determine the protein requirements of brook trout at two temperature regimes (15°C and 19°C)
- To compare the nutrient utilisation of brook trout at two different temperatures (15°C and 19°C) reflecting optimum and summer temperatures in south-eastern Australia

Chapter 3

- To evaluate the effect of dietary carbohydrate level (0-26%) on the key hepatic enzyme activities and histological feature of brook trout at two temperatures (15°C and 19°C)

Chapter 4

- To determine the effect of level of carbohydrate and lipid at optimum level of protein on the growth at two temperatures (15°C and 19°C)

Chapter 5

- To determine the maintenance dietary protein and energy requirement of brook trout at two temperatures (15°C and 19°C) by factorial modelling
- Determine the total dietary protein and energy requirement of brook trout at optimum (15°C) to high temperatures (19°C) by factorial modelling
- To develop diet specification table of brook trout on the basis of their protein and energy requirement

1.12. Notes on this study

Three major experiments were conducted to obtain data for this study. In chapter 5, data for the factorial modelling was provided by three separate experiments. The experimental chapters have been prepared as manuscripts for publication in peer-reviewed journals. Therefore, some content of this thesis, particularly the introduction and materials and methods for research chapters may be repeated.

Chapter 2 and Chapter 3 have been prepared from one experiment, thus general methods used for fish handling and sampling are referred in chapter 2 in order to avoid further repetition.

Chapter 5 mainly used the data to determine protein and energy requirements using factorial models and developed a theoretical feeding for rainbow trout. In order to keep focusing on protein and energy requirement model, model of weight gain was not included.

Chapter 6, the general discussion, the models of weight gain against feed intake, digestible protein and energy intake have been developed by using the data from Chapter 5.

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CHAPTER 2

EFFECT OF DIFFERENT PROTEIN LEVELS ON GROWTH PERFORMANCE AND FEED
UTILISATION OF BROOK TROUT, *SALVELINUS FONTINALIS* (MITCHILL, 1814) AT
TWO TEMPERATURES

2.1. Abstract

A twelve-week feeding trial was conducted to determine the optimum dietary protein requirement of brook trout, *Salvelinus fontinalis* at 15°C and 19°C. Twelve iso-energetic (22 MJ·kg⁻¹) and iso-lipidic (23%) diets (36 to 58% protein at 2% increments) were prepared. Fish were fed 2% of body weight per day, equally divided into two rations. Specific growth rate (SGR, %·d⁻¹), feed utilisation indices (FI, FER, PPV, PLV and PEV), apparent digestibility of diet (AD_{DM}) and protein (AD_{CP}) were significantly higher at optimum temperature (15°C). Increasing PPV with increasing dietary carbohydrate and with decreasing dietary protein content was due to protein sparing of carbohydrate. Piecewise regression (broken line) model between the SGR and digestible dietary protein level revealed that the digestible dietary protein requirement of brook trout was 44% and 40% at 15°C and 19°C, respectively, and it was 50% dietary crude protein at both temperatures. When PPV (digestible protein retention basis) was modelled by broken line, the digestible protein requirement of brook trout was 39% and 35% at 15°C and 19°C, respectively, and when expressed as dietary crude protein, the requirement was 44% at both temperatures. Temperature affected the digestible protein requirement, but not the crude protein requirement. A reduction of dietary protein content balanced by increased gelatinised carbohydrate might be useful to improve protein efficiency for growth at 15°C and 19°C, however, growth and feed efficiency was lower at elevated temperature.

2.2. Introduction

Protein is the most important macro-nutrient in fish feeds and fish require more dietary protein than other vertebrates (Murai, 1992; Luo et al., 2004). Prior to more detailed investigations of amino acid requirements, the crude protein requirement is necessary to determine the level of nutrient required to provide the necessary amount of amino acids (Hauler and Carter, 2001; Ward et al., 2003). This knowledge is also required to further investigate the replacement of protein and non-protein energy sources to achieve effective growth. When the diet contains insufficient amount of non-protein energy to crude protein, protein is catabolised for energy need (Sedgwick, 1979). Inadequate dietary protein can reduce or cease growth of fish (Mohanta et al., 2008). When dietary protein level exceeds the requirement, growth may remain constant or be reduced (Jauncey, 1982). Above the optimum, excess protein may be utilised for energy, increasing feed cost and ammonia excretion (Meyer and Fracalossi, 2004).

Temperature affects the growth, nutrient utilisation and nutrient digestibility of fish (Brett, 1979; Jobling, 1997; Azevedo et al., 1998). Fish growth rates increase with increasing water temperature until the maximum growth is reached at an optimal temperature and then decrease as the upper thermal tolerance is reached (Brett, 1979; Jobling, 1997; Katersky and Carter, 2007). Within the thermal tolerance range, feed intake increases with temperature to peak at an optimum temperature and then decreases (Imsland et al., 2006; Katersky and Carter, 2007). The optimum temperature for feed intake is generally a few degrees higher than that of growth; and peaks closer to the upper thermal limit (Jobling, 1994; Jobling, 1997). The effect of temperature on apparent nutrient digestibility in salmonids, however, is not clear (Olsen and Ringø, 1998; Bendiksen et al., 2003; Ng et al., 2004). Some authors found decreased nutrient digestibility at reduced temperature (Watanabe et al., 1996; Azevedo et al., 1998; Olsen and Ringø, 1998; Bendiksen et al., 2003; Miegel et al., 2010), whilst others found no effect of temperature on nutrient digestibility (Windell et al., 1978; Austreng et al., 1980).

There is controversy about the effect water temperature on protein requirements of fish (NRC, 1993; Wilson, 2002; Singh et al., 2009). The optimum protein level for chinook

salmon, *Oncorhynchus tshawytscha* has been shown to be dependent upon water temperature (De Long et al., 1958), where the protein requirement was 40% of the diet at 8°C and 55% at 15°C. In contrast, the optimum protein requirement of rainbow trout, *Oncorhynchus mykiss* was 35% and it was not affected by water temperature ranging from 9°C to 18°C (NRC, 1993). Peres & Oliva-Teles (1999) found that there was no effect of temperature on the protein requirement for European sea bass, *Dicentrarchus labrax* reared at either 18°C or 25°C temperature, but protein utilisation was higher at lower temperatures. Optimum protein requirement of European sea bass was 40% at 20°C whereas, there was no clear optimum at 15°C although daily protein requirement was lower at 15°C than at 20°C (Hidalgo and Alliot, 1988).

Numerous studies have been conducted on dietary protein requirement of rainbow trout (Murai, 1992; Kim, 1997), Atlantic salmon (Wiggs et al., 1994) and brown trout (Arzel et al., 1995) however, research on the optimum protein requirement for brook trout is limited (Jobling et al., 2010). Because information about diet utilisation and nutrient requirements are not available, brook trout have generally been fed diets formulated for rainbow trout (Jobling et al., 2010). Brook trout have more efficient protein retention and feed utilisation than rainbow trout (Rasmussen and Ostefeld, 2000) and the growth of brook trout has been shown to be higher than that of rainbow trout (Okumuş et al., 1999). Brook trout inhabit water ranging from 0°C to 20°C (Power, 1980), although their preferable temperature has been shown to range from 11 to 19°C (Graham, 1949; Clements, 1988). Optimum temperature for growth should not exceed 15.6°C (Raleigh, 1982). However, in Tasmania where brook trout are normally farmed summer temperatures are increasing and often reach to about 19 to 20°C (Miller et al., 2006; Ng et al., 2010; Lough and Hobday, 2011). In light of these results, we aimed to investigate the optimum dietary protein requirement of brook trout at two different temperatures reflecting optimum and summer culture temperatures in south-eastern Australia.

2.3. Materials and Methods

2.3.1. Experimental diets

Twelve isoenergetic (22 MJ·kg⁻¹) and isolipidic (23%) diets were formulated with varying protein levels (36 to 58%) increasing at 2% intervals (Table 2.1). Gelatinised

maize starch (BO11C) was added to balance the energy. The main protein source (fish meal) and fish oil were supplied by Skretting (Cambridge, Tasmania, Australia). All dry ingredients were mixed thoroughly by a Brice mixer (Model: VFM – 20C, Brice Australia Pty Ltd.) and approximately 12% water was added. Ytterbium oxide was added as an inert marker to the diet at the start of feed preparation. The 2 mm pellets were made using a California Laboratory Pellet Mill (California Laboratory Pellet Mill Co., San Francisco, USA) and dried (Oven, Model: 68732-1, Forma Scientific, Division of Mallinckrodt. INC. Marietta, Ohio, USA) to below 10% moisture content and stored at 2°C.

2.3.2. Experimental system and design

The experiment was conducted in two freshwater recirculation systems at the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), University of Tasmania, Launceston, Australia. Each system had twelve 300L tanks. One system was maintained at 15°C and the other at 19°C. Twelve diets were each allocated to one tank of fish, in both temperature systems. Water temperature of each system was controlled by a heat chiller unit and temperature was recorded hourly by a temperature data logger (HOBO[®] Pendant, UA-002-XX, Onset Computer Corporation, Pocasset, USA). The average temperatures (mean \pm SD) were 14.82 \pm 0.40°C and 19.42 \pm 0.50°C respectively. Water quality parameters (dissolved oxygen, pH, ammonia, nitrite and nitrate) were recorded daily and maintained within the limit for salmonids. Dissolved oxygen was recorded above 90% for this experiment. Water in each of experimental system was treated with biofilter and UV light. Water flow for each tank was equally maintained so that less inter-tank variation in dissolved oxygen, temperature and other water quality can occur.

Table 2.1. The ingredient and chemical composition of experimental feeds (g·kg⁻¹)

Ingredient Inclusion	P36	P38	P40	P42	P44	P46	P48	P50	P52	P54	P56	P58
Fish meal ¹	395	424	453	482	511	541	570	599	628	658	687	716
Wheat gluten ²	100	100	100	100	100	100	100	100	100	100	100	100
Fish oil ¹	154	150	147	144	140	137	133	130	126	123	119	116
Pregelatinised maize starch ³	284	259	233	207	182	155	130	104	79	52	27	1
Vitamins ⁴	15	15	15	15	15	15	15	15	15	15	15	15
Minerals ⁵	15	15	15	15	15	15	15	15	15	15	15	15
Binder CMC ⁶	10	10	10	10	10	10	10	10	10	10	10	10
Stay C ⁷	15	15	15	15	15	15	15	15	15	15	15	15
Choline chloride ²	1	1	1	1	1	1	1	1	1	1	1	1
Monobasic calcium phosphate ⁶	10	10	10	10	10	10	10	10	10	10	10	10
Ytterbium oxide ⁶	1	1	1	1	1	1	1	1	1	1	1	1
<i>Chemical Composition (dry matter)</i>												
Dry matter	926.5	921.1	919.9	927.1	921.0	913.6	924.1	917.8	915.4	915.5	914.3	923.5
Crude protein	377.7	385.8	411.1	430.7	446.5	461.8	490.7	502.4	532.4	555.6	572.2	601.0
Crude lipid	231.4	233.5	237.1	240.5	238.3	243.7	245.1	241.4	238.7	233.9	235.4	242.5
Ash	105.2	106.4	107.5	109.4	110.7	116.0	117.0	119.3	124.7	131.1	144.7	145.8
NFE ^a	285.7	274.3	244.4	219.4	204.6	178.5	147.2	136.9	104.2	79.4	47.7	10.7
Gross energy (MJ·kg ⁻¹)	22.72	22.66	23.31	23.00	23.22	23.02	22.45	22.63	22.81	23.26	22.35	22.70
P:E (g·MJ ⁻¹)	16.6	17.0	17.6	18.7	19.2	20.1	21.9	22.2	23.3	23.9	25.6	26.5

^aNFE (dry matter) = 100 – (Crude protein% + Crude lipid % + Ash%); ¹Skretting Australia, Cambridge, Tasmania, Australia; ²MP Bio-medicals, LLC, 29525 Fountain Pkwy, Solon, OH 44139, France; ³National Starch Pty. Ltd., 170 Epping Road, Lane Cove 2066, NSW, Australia; ⁴ Vitamin premix (mg·kg⁻¹ of mixture) = Vitamin A acetate (ICN), 7.50; Vitamin D3 powder (ICN), 9.00; Rovimix E50, 150.00; Menadone sodium bisulphate, 3.00; Riboflavin, 6.00; Calcium D-pantothenate, 32.68; Nicotinic Acid, 15.00; Vitamin B12, 0.015; d-Biotin, 0.225; Folic Acid, 1.50; Thiamin HCL, 1.68; Pyridoxine HCL, 5.49; Myo-Inositol, 450.00; α -cellulose, 817.91; Stay-C, 150.00; ⁵Mineral premix (mg·kg⁻¹ of mixture) = CuSO₄ 5H₂O (cupric sulphate), 35.37; FeSO₄ 7H₂O (ferrous sulphate), 544.65; MnSO₄ H₂O (manganese sulphate), 92.28; Na₂SeO₃ (sodium selenate), 0.99; ZnSO₄ 7H₂O (zinc sulphate), 197.91; KI (potassium iodide), 2.16; CoSO₄ 7H₂O (cobalt sulphate), 14.31; α -cellulose, 612.33; ⁶Sigma-Aldrich, Castle Hill, NSW, Australia; ⁷L-ascorbyl-2-polyphosphate (Roche Vitamins Australia, Frenchs Forest, NSW, Australia).

2.3.3. *Experimental fish and growth experiment*

Fish were provided by Mountain Stream Fishery (Nunamara, Tasmania, Australia). Fish were held 2000L tank and fed daily before using for experiment. Fish were randomly allocated to the 24 experimental tanks (26 fish-tank⁻¹) and fed a 2 mm commercial rainbow trout diet (Spectra SS, Skretting, Cambridge, Tasmania, Australia) at 1% of body weight a day for one week of acclimation to the experimental systems. Temperature was maintained at 15°C in one system and slowly increased (1°C·d⁻¹) to 19°C in the other system. Before starting the experiment, individual fish were weighed (g) and length was measured (cm). Twelve fish were randomly selected from each temperature system and killed by overdose (400 ppm) of anaesthetics (Aqui-S, AQUI-S New Zealand Ltd) to determine the initial whole-body chemical composition.

Twenty five fish (29.45 ± 3.25 g·fish⁻¹) per tank were grown in the experimental system for 12 weeks. During the growth experimental the fish were fed the experimental diet at a uniform ration of 2% of body weight which was equally divided in two meals (9:30 and 15:30) daily. Every 3 weeks fish were fasted for 24 h, anaesthetised with Aqui-S (100 ppm) and the bulk weight of fish in each tank was recorded. Rations were recalculated according to biomass for the following 20 d period. Mortality was recorded during the experimental period and the ration was adjusted without replacing fish. At the end of the experiment, individual fish were weighed (g). Five fish from each tank were euthanised by an overdose (400 ppm) of the anaesthetic (Aqui-S) and frozen (-20°C) for later determination of body composition. Twelve fish were returned to each tank for digestibility measurements.

2.3.4. *Apparent digestibility*

The remaining 12 fish in each treatment were fed the same experimental feeds for 7 d. During the faecal collection day a staggered feeding regimes was taken in order to maintain the time consistency between last feeding and stripping. Fish were anaesthetised and faeces were collected by stripping (Percival et al., 2001) from the distal part of the small intestine 4 h after feeding. Faeces were frozen, freeze dried and stored at -20°C for chemical analysis. Apparent digestibility coefficients of diet and protein were calculated using the formula:

Apparent digestibility of dry matter (AD_{DM}) =

$$100 - 100 \cdot (\% \text{ marker in feed} \cdot \% \text{ marker in faeces}^{-1}) \quad (\text{De Silva and Anderson, 1995}).$$

Apparent digestibility of protein (AD_{CP}) =

$$100 - [100 \{(\% \text{ marker in feed} \cdot \% \text{ marker in faeces}^{-1}) \cdot (\% \text{ nutrient in faeces} \cdot \% \text{ nutrient in feed}^{-1})\}] \quad (\text{Maynard and Loosli, 1969}).$$

2.3.5. Chemical analysis

Fish and feed samples were autoclaved (Williams et al., 1995) before freeze drying to constant weight, then homogenised for chemical analysis. Dry matter, ash, crude protein were analysed according to AOAC standard procedures (AOAC, 2005). Crude lipid was determined according to Bligh and Dyer (1959), crude protein by Kjeldahl method and gross energy through combustion in a calorimetric bomb (Gallenkamp Autobomb). To analyse the ytterbium, freeze dried feed and faecal samples were homogenised then digested in 2 ml concentrated HNO_3 at $90^\circ C$ for 3 h. The digests had 10ml of 10,000 ppm KNO_3 added and then diluted (1:100, v/v) with distilled water. Ytterbium content was analysed by Flame Atomic Absorption Spectrometry (XploraAA, GBC Scientific Equipment, Australia) using nitrous oxide – acetylene flame (lamp current: 5 mA, wavelength: 398.8 nm and a slit width of 0.2 nm).

2.3.6. Calculation

The nutrient efficiency and performance indices were determined as follows:

Specific growth rate (SGR) ($\% \cdot d^{-1}$)

$$= 100 \cdot (\ln \text{ final weight} - \ln \text{ initial weight}) \cdot \text{day}^{-1} \quad (1)$$

Feed efficiency ratio (FER) ($g \cdot g^{-1}$)

$$= \text{weight gain} \cdot \text{dry feed fed}^{-1} \quad (2)$$

Productive protein value (PPV) (%)

$$= (\text{protein gain in the body (wet)} \cdot \text{crude protein fed}^{-1}) \cdot 100 \quad (3)$$

Productive protein value (digestible protein fed basis) (PPV_D) (%)

$$= (\text{protein gain in the body (wet)} \cdot \text{digestible protein fed}^{-1}) \cdot 100 \quad (4)$$

Productive lipid value (PLV) (%)

$$= (\text{lipid gain in the body (wet)} \cdot \text{lipid fed}^{-1}) \cdot 100 \quad (5)$$

Productive energy value (PEV) (%)

$$= (\text{energy gain in the body (wet)} \cdot \text{energy fed}^{-1}) \cdot 100 \quad (6)$$

2.3.7. Statistical analysis

The experiment was designed for regression modelling without replication (Shearer, 2000). While water flow, aeration in each tank was maintained as equal as possible to reduce the inter-tank variation, individual variation may occur as a result of biological factors. It is unrealistic to analyse every single fish in that population, so in order to retain statistical confidence in this experiment, random samples were taken from the underlying biological population (biological replicates) and pooled for analysis. Thus in a pooled sample, there is no biological variance that can be used in the statistics to calculate a p-value. Results of weight and survival are presented as mean \pm SD. The growth performance and nutritional indices measured and were expressed as mean \pm SD. The effect of dietary protein levels of diet on growth performance and nutritional utilisation indices were analysed by linear regression separately for both temperatures. To determine the effect of temperature on growth performance and nutritional indices, differences in the slope between two temperatures were analysed by ANCOVA. All data were analysed by using SPSS software (version 17). Protein requirement of fish was determined by piecewise regression model (Ryan and Porth, 2007) using SGR ($\% \cdot \text{d}^{-1}$) and productive protein value (PPV, %) with digestible protein in diet.

2.4. Results

2.4.1. Growth performance

Initial weight (mean \pm SD) of fish was 29.68 ± 4.16 g and 28.70 ± 4.91 g for 15°C and 19°C, respectively. Survival (mean \pm SD) in this experiment was $99.15 \pm 0.69\%$. Total feed intake at 15°C significantly increased with dietary protein level ($R^2=0.67$, $F_{1,10}=20.031$, $P=0.001$) and was described by the equation $\text{FI} = 0.345\text{CP}(\%) + 80.995$, while no significant differences were found at 19°C ($R^2=0.01$, $F_{1,10}=0.125$, $P=0.731$). Feed intake was significantly higher ($F_{1,11}=44.489$, $P<0.001$) at 15°C than 19°C (Table

2.2). There was a significant linear relationship in SGR (%.d⁻¹) with increasing dietary protein level at both temperatures, 15°C ($R^2=0.65$, $F_{1,10}=18.191$, $P=0.002$) and 19°C ($R^2=0.50$, $F_{1,10}=9.912$, $P=0.010$) and a significantly higher SGR ($F_{1,11}=115.91$, $P<0.001$) was determined in fish reared at 15°C than 19°C (Table 2.2 and Fig. 2.1). Specific growth rate at 15°C can be explained by the equation $SGR = 0.007CP(\%) + 1.566$ while at 19°C it was $SGR = 0.008CP(\%) + 1.400$.

2.4.2. Feed utilisation

Feed efficiency ratio (FER) significantly increased with increasing dietary protein level at both temperatures 15°C ($R^2=0.71$, $F_{1,10}=23.995$, $P=0.001$) and 19°C ($R^2=0.37$, $F_{1,10}=5.866$, $P=0.036$). At 15°C, FER was explained by the equation $FER = 0.006CP(\%) + 0.979$ while at 19°C it was $FER = 0.004CP(\%) + 0.981$. Generally, fish at 15°C utilised the feed better ($F_{1,11}=39.38$, $P<0.001$) than fish reared at 19°C (Table 2.2). There was a significant negative linear relationship between the productive protein value (PPV) and dietary protein level at both temperatures 15°C ($R^2=0.79$, $F_{1,10}=38.508$, $P<0.001$) and 19°C ($R^2=0.95$, $F_{1,10}=195.725$, $P<0.001$) (Fig. 2.2). At 15°C, PPV was explained by the equation $PPV = -0.606CP(\%) + 74.495$ while at 19°C it was $PPV = -0.601CP(\%) + 68.705$. At 15°C, PPV was significantly higher ($F_{1,11}=93.684$, $P<0.001$) than at 19°C (Table 2.2 and Fig. 2.2). There was a significant negative relationship between productive lipid value (PLV) and dietary protein level of diet at both temperatures 15°C ($R^2=0.58$, $F_{1,10}=13.728$, $P=0.004$) and 19°C ($R^2=0.75$, $F_{1,10}=29.749$, $P<0.001$). At 15°C PLV was explained by the equation $PLV = -0.488CP(\%) + 95.157$ and at 19°C PLV was explained by the equation $PLV = -0.710CP(\%) + 95.465$. At the lower temperature, PLV was significantly higher ($F_{1,11}=125.787$, $P<0.001$) than at 19°C (Table 2.2). Productive energy value (PEV) was not significantly related to dietary protein at either temperature; 15°C ($R^2=0.01$, $F_{1,10}=0.112$, $P=0.745$) or 19°C ($R^2=0.26$, $F_{1,10}=3.543$, $P=0.089$). The mean PEV at 15°C was 47.47 ± 2.21 and at 19°C it was 41.15 ± 2.05 . However, at the lower temperature PEV was significantly higher ($F_{1,11}=51.956$, $P<0.001$) than at high temperature (Table 2.2).

Table 2.2. Growth performance and feed efficiency of brook trout, *Salvelinus fontinalis* fed experimental diets over 12 weeks.

	Temp	P36	P38	P40	P42	P44	P46	P48	P50	P52	P54	P56	P58	Sig
Initial weight (g)	15°C	29.83	30.69	28.75	30.64	30.28	29.62	29.06	28.35	28.70	30.43	29.32	30.45	
		±1.16	±5.31	±4.61	±4.04	±4.70	±1.98	±5.29	±4.40	±3.29	±3.37	±4.25	±5.23	
	19°C	29.28	27.49	30.04	30.20	29.25	29.58	28.34	30.10	27.78	28.93	28.85	29.58	
		±4.65	±4.75	±4.27	±4.23	±6.25	±3.50	±5.96	±3.86	±3.31	±4.00	±3.86	±4.25	
Final weight (g)	15°C	142.67	142.22	145.42	155.50	155.25	153.60	155.95	160.39	158.22	164.99	168.93	162.45	
		±18.34	±12.08	±25.36	±22.39	±29.58	±24.67	±26.61	±14.37	±18.72	±28.05	±29.77	±27.38	
	19°C	122.70	121.38	124.09	136.84	141.04	134.67	144.73	141.68	137.59	121.30	127.32	140.11	
		±23.15	±16.15	±21.78	±26.55	±22.72	±25.49	±37.81	±23.17	±18.09	±23.08	±23.25	±26.94	
Survival (%)	15°C	100	100	100	100	100	100	100	100	100	96	100	100	
	19°C	100	96	96	100	96	100	100	96	100	100	100	100	
Total feed intake (g·fish ⁻¹)	15°C	91.11	95.19	93.87	97.55	98.44	98.84	97.59	97.24	97.58	102.30	101.71	99.69	*
	19°C	85.13	81.92	85.26	89.20	89.74	87.77	91.99	92.66	87.47	78.90	83.63	91.35	
SGR (%·d ⁻¹)	15°C	1.82	1.78	1.89	1.89	1.90	1.91	1.95	2.02	1.98	1.97	1.96	1.95	*
	19°C	1.67	1.66	1.65	1.76	1.83	1.76	1.90	1.86	1.85	1.82	1.81	1.81	
FER	15°C	1.24	1.17	1.24	1.28	1.27	1.25	1.30	1.36	1.33	1.32	1.37	1.32	*
	19°C	1.10	1.15	1.10	1.20	1.25	1.20	1.27	1.20	1.26	1.21	1.20	1.21	
PPV (%)	15°C	51.93	47.04	47.89	53.05	49.78	45.39	45.53	46.21	41.39	39.52	38.50	38.33	*
	19°C	43.87	45.22	44.32	44.61	42.91	41.24	38.75	38.92	37.31	34.67	33.42	32.58	
PLV (%)	15°C	84.14	76.63	71.03	70.66	71.48	72.16	68.23	72.87	69.48	69.33	68.35	66.07	*
	19°C	73.04	72.13	61.73	63.15	65.48	57.62	59.41	58.85	56.21	56.92	56.12	55.46	
PEV (%)	15°C	48.33	46.60	45.85	46.79	46.80	46.17	49.68	50.71	48.56	42.86	50.45	46.85	*
	19°C	40.54	43.09	43.44	45.48	40.82	38.71	42.04	40.76	39.37	38.81	39.53	41.15	
AD _{DM} (%)	15°C	77.81	79.87	79.21	78.06	78.90	78.03	76.53	78.33	77.94	77.24	76.38	78.10	*
	19°C	67.17	69.05	69.20	66.67	67.31	68.52	68.56	66.38	67.42	67.15	67.33	67.27	
AD _{CP} (%)	15°C	87.11	87.25	87.57	87.00	88.13	86.81	88.66	88.76	87.11	88.04	88.45	88.04	*
	19°C	81.50	82.56	80.27	80.69	80.07	81.58	80.90	79.49	79.48	81.30	81.97	81.45	

Total feed intake, dry matter basis (g·fish⁻¹) = feed consumed over 12 weeks; Specific growth rate (SGR, %·d⁻¹) = 100 x (ln final weight - ln initial weight)·day⁻¹; Feed efficiency ratio (FER) = weight gain·dry feed fed⁻¹; Productive protein value (%PPV) = (protein gain in the body (wet)·crude protein fed⁻¹) x 100; Productive lipid value (%PLV) = (lipid gain in the body (wet)·lipid fed⁻¹) x 100; Productive energy value (%PEV) = (energy gain in the body (wet)·energy fed⁻¹) x 100; Apparent dry matter digestibility (AD_{DM}) = 100 - 100 x (% Ytterbium in feed - % Ytterbium in faeces⁻¹); Apparent digestibility of protein (AD_{CP}) = 100 - 100 {(% marker in feed - % marker in faeces⁻¹) x (% nutrient in faeces· % nutrient in feed⁻¹)}. * indicates statistical significance at $P = 0.001$ between two temperatures when analysed by ANCOVA.

2.4.3. Apparent digestibility

Apparent digestibility of dry matter (AD_{DM}) was not significantly affected by dietary protein level at either temperature, 15°C ($R^2=0.341$, $F_{1,10}=5.174$, $P=0.056$) or 19°C ($R^2=0.149$, $F_{1,10}=1.746$, $P=0.216$). The mean AD_{DM} (\pm SD) at 15°C was 78.03 ± 1.01 and at 19°C was 67.67 ± 0.92 . At 15°C, AD_{DM} was significantly higher ($F_{1,11}=1013.362$, $P<0.001$) than 19°C (Table 2.2). At both temperatures, the apparent digestibility of protein (AD_{CP}) of diet was not significantly affected by dietary protein level ($R^2=0.263$, $F_{1,10}=3.577$, $P=0.088$ at 15°C; $R^2=0.006$, $F_{1,10}=0.058$, $P=0.814$ at 19°C). The mean AD_{CP} (\pm SD) at 15°C was $87.74 \pm 0.68\%$ and at 19°C was $80.94 \pm 0.97\%$. At 15°C AD_{CP} was significantly higher ($F_{1,11}=334.870$, $P<0.001$) than 19°C (Table 2.2).

2.4.4. Proximate composition

The proximate composition of the experimental fish was affected by dietary protein level; this was markedly evident for crude lipid and moisture content (Table 2.3). A significant positive relationship existed between the crude protein content of fish and dietary protein level at 19°C ($R^2=0.38$, $F_{1,10}=6.039$, $P=0.034$) but at 15°C no significant relationship was determined ($R^2=0.22$, $F_{1,10}=2.785$, $P=0.126$). Significantly higher protein content ($F_{1,11}=24.356$, $P<0.001$) was determined for fish at 15°C than at 19°C (Table 2.3). There was a significant negative relationship between the crude lipid content of fish and dietary protein content of diet at both temperatures ($R^2=0.81$, $F_{1,10}=41.933$, $P<0.001$ at 15°C; $R^2=0.69$, $F_{1,10}=22.063$, $P<0.001$ at 19°C). Significantly higher crude lipid ($F_{1,11}=61.565$, $P<0.001$) was determined for fish at 15°C than at 19°C (Table 2.3). There was a significant positive relationship ($R^2=0.52$, $F_{1,10}=10.849$, $P=0.008$ at 15°C; $R^2=0.47$, $F_{1,10}=8.851$, $P=0.014$ at 19°C) between the moisture content of fish ($680-710 \text{ g}\cdot\text{kg}^{-1}$) and dietary protein content at both temperatures. There was significantly higher moisture content ($F_{1,11}=75.12$, $P<0.001$) at 19°C than at 15°C (Table 2.3). Ash content of fish ($\text{g}\cdot\text{kg}^{-1}$) was not significantly different with dietary protein at both temperature conditions ($R^2=0.01$, $F_{1,10}=0.074$, $P=0.791$ at 15°C; $R^2=0.06$, $F_{1,10}=0.637$, $P=0.443$ at 19°C) but significantly higher ($F_{1,11}=34.739$, $P<0.001$) at 15°C (Table 2.3) and the average ash content was 31.7 ± 3.4 and 24.2 ± 1.7 at 15°C and 19°C, respectively.

Table 2.3. Proximate composition, g·kg⁻¹ wet weight, of brook trout, *Salvelinus fontinalis* fed experimental diets over 12 weeks at 15°C and 19°C.

	Temp	P36	P38	P40	P42	P44	P46	P48	P50	P52	P54	P56	P58
Crude protein	15° C	158.60	155.93	158.62	174.76	172.04	165.67	169.54	168.97	164.85	165.56	165.81	171.27
	19° C	154.32	155.15	156.67	161.68	156.13	160.38	153.19	162.97	159.64	160.58	160.44	162.51
Crude lipid	15° C	141.91	137.91	125.34	123.23	124.44	129.41	120.32	121.51	117.61	116.11	111.50	114.11
	19° C	134.62	130.14	118.15	115.14	114.32	107.50	106.81	108.43	100.01	102.20	102.10	109.41
Moisture content	15° C	684.10	682.81	687.10	687.71	695.70	696.01	689.53	689.39	698.50	711.10	702.20	693.14
	19° C	697.90	697.55	714.20	693.40	711.72	716.55	718.33	711.62	723.22	718.53	718.12	713.33
Ash content	15° C	32.70	30.20	33.63	33.34	28.14	27.55	33.71	39.71	31.51	27.27	31.31	31.01
	19° C	26.50	24.71	23.02	23.90	23.12	25.90	24.14	23.21	22.80	27.40	21.60	23.81
Gross energy (MJ·kg ⁻¹)	15° C	8.33	8.43	8.14	7.99	8.12	8.05	8.16	8.07	7.97	7.34	7.88	7.71
	19° C	7.85	7.98	7.32	8.18	7.31	7.16	7.20	7.34	6.95	7.19	7.10	7.39

Initial group (15°C): moisture content, 744.23, crude protein, 159.60, crude lipid, 84.10, ash content, 41.21, gross energy (MJ·kg⁻¹), 6.31; (19°C): moisture content, 745.90, crude protein, 165.10, crude lipid, 72.71, ash content, 50.51, gross energy (MJ·kg⁻¹), 6.15. Chemical analysis were performed by pooling sample (n=5).

2.4.5. Estimation of protein requirement

The effect of dietary protein content on fish growth and nutrient efficiencies was determined at the two temperatures. By piecewise regression modelling of digestible protein in the diet and SGR of fish, the digestible dietary protein requirement of brook trout was determined to be 44% and 40% at 15°C ($R^2=0.87$, $P<0.001$, $n=12$) and 19°C ($R^2=0.70$, $P<0.001$, $n=12$), respectively (Fig. 2.1). These values of optimum digestible dietary protein came from 50.2% and 49.2% of total crude protein at 15°C and 19°C, respectively. When PPV_D on a dietary digestible protein level was modelled, the dietary digestible protein requirement of brook trout was determined to be 39% and 36% at 15°C ($R^2=0.84$, $P<0.001$, $n=12$) and at 19°C ($R^2=0.95$, $P<0.001$, $n=12$), respectively (Fig. 2.2). These values of digestible protein came from 43.5% and 44.3% of total crude dietary protein at 15°C and at 19°C, respectively.

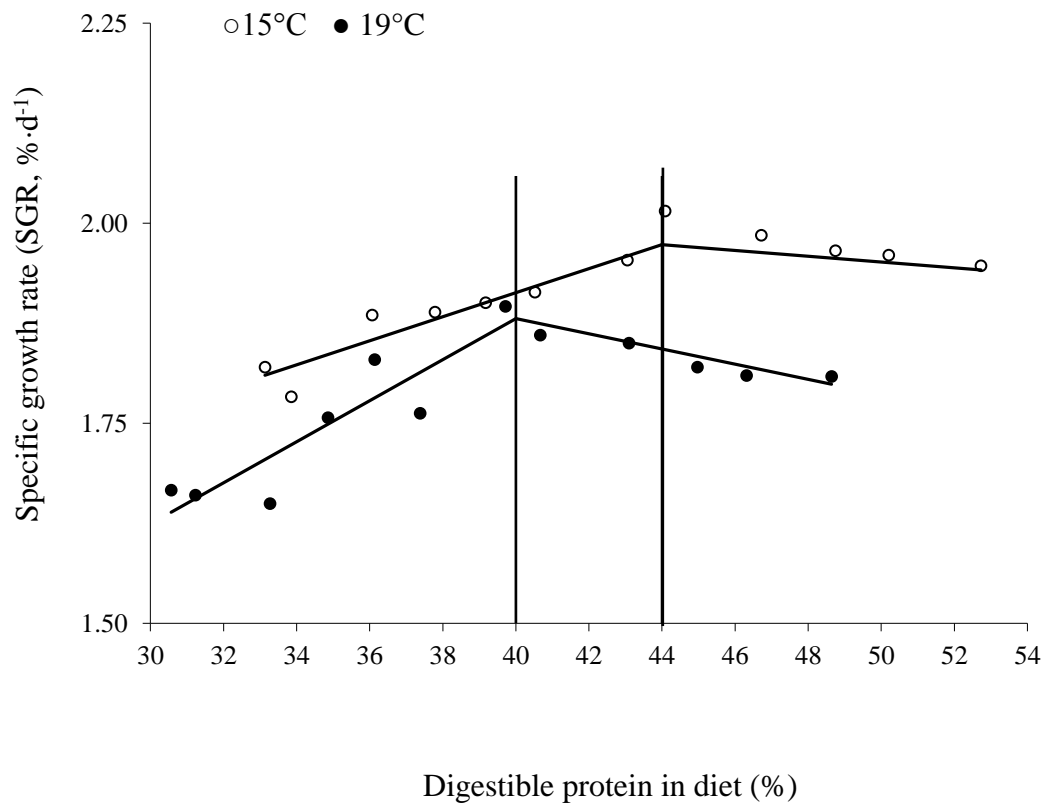


Figure 2.1. Optimum digestible dietary protein requirements for maximum growth of brook trout, *Salvelinus fontinalis* by using piecewise regression model. At 15°C (○) the relationship was described by $SGR = 0.015DP(\%) + 1.31$ and $SGR = -0.004DP(\%) + 2.13$ ($R^2 = 0.92$, $P < 0.001$, $n = 12$). The optimum digestible protein requirement was determined to be 44%. At 19°C (●) the relationship was described by $SGR = 0.026DP(\%) + 0.85$ and $SGR = -0.010DP(\%) + 2.26$ ($R^2 = 0.70$, $P < 0.001$, $n = 12$). The optimum digestible protein requirement was determined to be 40%.

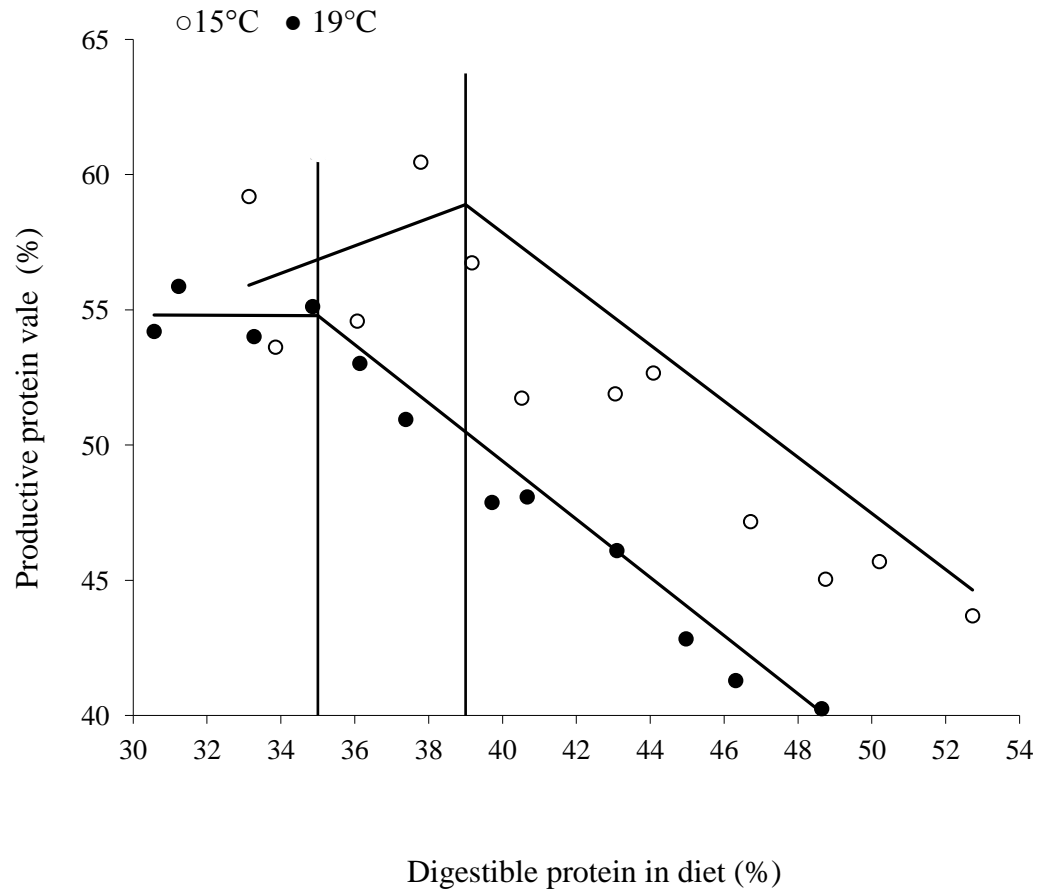


Figure 2.2. Optimum digestible dietary protein requirement for maximum protein utilisation of brook trout, *Salvelinus fontinalis* using piecewise regression model. At 15°C (○) the relationship was described by $PPV_D = 0.509DP(\%) + 39.03$ and $PPV_D = -1.038DP(\%) + 99.37$ ($R^2=0.80$, $P<0.001$, $n=12$). The optimum digestible protein requirement was 39%. At 19°C (●) the relationship can be described by $PPV_D = -0.004DP(\%) + 54.94$ and $PPV_D = -1.076DP(\%) + 92.45$ ($R^2=0.98$, $P<0.001$, $n=12$). The optimum digestible protein requirement was 35%.

2.5. Discussion

This is the first study to examine the effects of increased temperature on the protein requirement of brook trout. All growth and nutrient efficiency parameters were higher at 15°C than at 19°C, and temperatures above the optimum level reduced growth performance of the fish (This study; Person-Le Ruyet et al., 2006; Katersky and Carter, 2007). Metabolic rate of fish is increased at high temperature (Jobling, 1997; Katersky and Carter, 2005). Dissolved oxygen (DO) level is reduced at high temperature, which may limit the growth potential at high temperature due to an inability of the respiratory system to provide oxygen to respiring tissue under high oxygen demand for increased metabolism (Jobling, 1997; Katersky and Carter, 2007). Consequently, it accelerates the stress of fish at high temperature and limits the ability of fish to consume feed (Katersky and Carter, 2007). This might be evident in brook trout that feed intake was lower at high temperature. The lower growth efficiency of brook trout at high temperature is related to reduction of FER at the higher temperatures (This study; Katersky and Carter, 2005).

In this study, productive protein value (PPV) decreased with increasing dietary protein level, and indicates that more protein was catabolised for energy when fish were fed on a high protein diet (Jobling and Wandsvik, 1983). Carbohydrate was added to balance the energy content of diet, thus the diet with lower dietary protein level contained higher level of carbohydrate. When diet contained lower protein level, amino acids were utilised effectively to meet requirement (Wilson, 2002; Oliva-Teles, 2012); once requirement were met additional protein or extra carbohydrate allowed protein to store as growth. Therefore, an increasing value of PPV with decreasing protein level indicated that a protein sparing effect may have occurred in this study. The protein sparing effect of carbohydrate has also been seen in Atlantic salmon (Hemre et al., 1995; Grisdale-Helland and Helland, 1997) and sea bass (Hidalgo and Alliot, 1988). In this present study protein utilisation was greater at 15°C than 19°C and was consistent with other fish species where protein utilisation was more efficient at close to optimum temperature (Peres and Oliva-Teles, 1999; Guerreiro et al., 2012).

The increased PLV in the current study with decreasing protein level may be due to lipid deposition arising from higher availability of non-protein energy (carbohydrate)

(Mohanta et al., 2008). The PEV value in this study was not significantly different, therefore indicating that brook trout may efficiently utilise energy from protein as well as non-protein sources or that carbohydrate is equally utilised to replace energy from lipid. However, the positive effect of dietary starch on protein efficiency, and no effect on energy efficiency indicated similar energetic availability of starch and lipid (Krogdahl et al., 2004).

In the present study, the apparent protein digestibility (AD_{CP}) was higher (89.5%) at 15°C and decreased to 81.3% with the increased temperature at 19°C. In rainbow trout, AD_{CP} was lower at 6°C (81%) than at 15°C (89%) (Azevedo et al., 1998), however, above 15°C AD_{CP} was shown to decrease at both 20°C and 25°C (Watanabe et al., 1996). In Atlantic salmon, protein digestibility was high (around 91%) when temperature ranged from 2°C to 8°C (Bendiksen et al., 2003). In this study, the apparent digestibility of dry matter was also higher (75.58%) at 15°C and decreased to 59.64% at 19°C. Arctic charr, *Salvelinus alpinus* showed lower digestibility of dry matter and all nutrients at 0.6°C than at 10°C (Olsen and Ringø, 1998). Apparent digestibility of dry matter increased with temperature from 73% at 6°C to 82% at 15°C (Azevedo et al., 1998). Lower digestibility at high temperature was probably due to reduced digestive enzymatic activity and shorter gut transit time at high temperature (Kaushik, 1986; Papoutsoglou and Lyndon, 2005), which still needs to be confirmed for this fish.

The proximate composition of cultured fish is normally affected by a range of exogenous and endogenous factors (Shearer, 1994). In the present study, no significant difference was found in whole-body protein content at 15°C with increasing dietary protein, however, Yang *et al.* (2003) reported a positive trend in whole-body protein content with dietary protein level in Cyprinids, *Spinibabus hollandi*. In this study, the increased lipid deposition in brook trout could be due to *de novo* lipidogenesis from carbohydrate (Brauge et al., 1994; Fernández et al., 2007; Enes et al., 2008). Carp whole-body lipid increased when fed higher dietary carbohydrate (Singh et al., 2006; Mohanta et al., 2007). When dietary lipid was set at a constant level, the whole-body lipid content increased with increasing dietary carbohydrate as an indication of lipid synthesis from carbohydrate (Yang et al., 2003). Moisture content of the whole-body increased with dietary protein level and moisture was inversely related to lipid content

of body tissues (This study; Shearer, 1994). However, both the protein and lipid content of fish decreased at high temperature and may be explained by increased energy demand to support increased metabolism, where fish could not consume enough energy to store protein or lipid (Shearer, 1994; Jobling, 1997; Katersky and Carter, 2007).

Table 2.4. Digestible protein requirement of brook trout at 15°C and 19°C.

Temperature	Parameter	Digestible protein requirement (% in diet)
15°C	SGR	44
	PPV	39
19°C	SGR	40
	PPV	35

Different protein levels within the diet had a significant effect on growth rate (SGR) at both temperatures. A piecewise regression model between the SGR and dietary digestible protein level revealed that the digestible protein requirement of brook trout was 44% and 40% at 15°C and 19°C, respectively (Table 2.4). These amounts of digestible protein were approximately 50% of dietary crude protein at both temperatures. The higher requirement for digestible protein was most likely due to the higher SGR at the optimum temperature. At a given ration, the scope for growth rate is reduced progressively at high temperatures due to a marked increase in energy requirements for maintenance (Jobling, 1994; Shearer, 1994). The reduced SGR at high temperature might be due to lower protein digestibility at high temperature. Application of this model to PPV (based upon digestible protein ingested) showed that digestible protein requirement of brook trout was 39% and 35% at 15°C and 19°C, respectively (Table 2.4). These digestible protein levels were equivalent to about 44% of dietary crude protein at both temperatures. Dietary protein requirements could be overestimated if growth is used to determine the protein requirements with no attention to protein utilisation (Hidalgo and Alliot, 1988). When weight gain was modelled, the protein requirement of sea bass was 50% and, when protein retention (PPV) was used as a criterion, the protein requirement was 40% (Hidalgo and Alliot, 1988). Higher dietary carbohydrate reduced the protein used for energy and increased protein utilisation. Basically, protein requirement of fish means that amount of protein required to meet the demand of amino acid for any species (Wilson, 2002). At lower protein level (44%) in the diet of brook trout, the protein may have been effectively used to meet this amino acid requirement and additional protein may be required to produce better growth. The dietary protein requirement obtained from this experiment was close Arctic charr and it was 44% (for best feed utilisation) to 54% (for best weight gain) (reviewed by Jobling et al., 1993). The protein requirements of brown trout was between 48 and 53%, when specific growth rate was used as a criterion (Arzel et al., 1995). The protein requirement for rainbow trout was 40-45% and for Arctic charr 36-43.6%, criterion used for those model was not mentioned (Tacon and Cowey, 1985).

2.6. Conclusion

Brook trout growth parameters and feed utilisation indices were better at optimum temperature of 15°C than at the higher temperature of 19°C which is often experienced during summer months. Pre-gelatinised carbohydrate showed a protein sparing effect in brook trout. The reduced digestible protein requirement at high temperature was due to a lower apparent protein digestibility at high temperature. However, temperature may not affect the crude protein requirements of fish (Wilson, 2002). Further investigation is needed to determine the cause of lower apparent protein digestibility at high temperature and to determine the upper capacity of brook trout to utilise carbohydrate to replace protein. Although the dietary crude protein requirement for brook trout was similar at optimum and at an elevated temperature under a 2% BW·d⁻¹ of feeding regimes, the digestible protein requirement was lower at elevated temperatures. Brook trout reared at 10°C and fed diet (crude protein 49%, lipid 23% and energy 22.9 MJ·kg⁻¹) lower to satiety showed SGR range 0.52 to 1.00 and FER 1.22 (Rasmussen and Ostensfeld, 2000). In this study brook trout fed gelatinised carbohydrate based diet (protein 37-60%, lipid 23% and 23 MJ·kg⁻¹) showed satisfactory growth (SGR 1.78-2.02 at 15°C and 1.65-1.90 at 19°C) and feed efficiency (FER 1.17-1.37 at 15°C and 1.10-1.27 at 19°C) at both temperature indicated that high potential of using gelatinised carbohydrate in both winter and summer feed. Increasing water temperatures present a current and future challenge for the global salmonid industry and further understanding of the effect of temperature on the availability of plant derived energy sources is warranted.

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CHAPTER 3

HEPATIC ENZYMATIC REGULATION AND HISTOLOGICAL FEATURE OF LIVER OF
BROOK TROUT, *SALVELINUS FONTINALIS* (MITCHILL, 1814) FED VARYING LEVEL OF
CARBOHYDRATE

3.1. Abstract

This study evaluated the effect of temperature and increasing levels of gelatinised maize starch balanced by decreasing protein content in iso-energetic diets on liver histology and intermediary carbohydrate metabolism of brook trout, *Salvelinus fontinalis*. The detailed experimental procedures and results for growth and nutrient utilisation have been described in chapter 2. A twelve-week feeding trial was conducted at 15°C and 19°C. Twelve iso-energetic (22 MJ·kg⁻¹) and iso-lipidic (23%) diets (0.1 to 28% gelatinised carbohydrate, energy balanced by increasing content of protein) were prepared. Fish were fed 2% of body weight per day, equally divided into two rations. Increasing levels of dietary gelatinised maize did not show any pathological symptoms in the liver; however, hypertrophic hepatocytes were found due to increased storage of glycogen. There was an increasing trend in hepatosomatic index (HSI) in brook trout fed higher levels of gelatinised carbohydrate. The activity of glycolytic enzyme (PK) was increased with increasing level of gelatinised carbohydrate and it was higher at 19°C. Neither temperature nor dietary gelatinised carbohydrate levels affected the lipogenic enzyme (G6PDH) activity. Higher levels of gelatinised carbohydrate reduced the protein catabolic enzyme (GDH) activity. Gelatinised maize starch can be used as good sources of non protein energy in the diet of brook trout particularly at elevated temperatures.

3.2. Introduction

Carnivorous fish have limited capacity to utilise carbohydrate as energy (Wilson, 1994; Hemre et al., 2002; Panserat et al., 2009), although different fish species have no discernible pattern of ability to utilise carbohydrate (Hemre et al., 2002; Enes et al., 2009). Dietary carbohydrate utilisation by fish depends carbohydrate complexity, origin, physical state, inclusion content, processing techniques and endogenous enzymatic activity of fish (Wilson, 1994; Stone, 2003; Krogdahl et al., 2005). Processing techniques such as extrusion improve the carbohydrate utilisation in fish by increasing gelatinisation of raw starch (Stone, 2003; Krogdahl et al., 2005). Gelatinisation breaks down the complex starch granule and increases the surface area, which leads to increase enzymatic action on starch (Bergot and Breque, 1983; Stone, 2003). The higher digestibility of gelatinised carbohydrate is documented in rainbow trout, *Oncorhynchus mykiss* (Inaba et al., 1963; Pieper and Pfeffer, 1980; Bergot and Breque, 1983), but lower digestibility of gelatinised carbohydrate with increasing inclusion level was found in Atlantic salmon, *Salmo salar* (Aksnes, 1995).

Hepatosomatic index (HSI), hepatic glycogen content and key enzyme activities of intermediary metabolism are currently used to determine the metabolic utilisation of dietary carbohydrate of fish (Hemre et al., 2002; Rawles et al., 2008; Enes et al., 2009). Hexokinase (HK) or glucokinase (GK, isomer of HK) facilitates the first reaction of glycolysis involving phosphorylation of glucose to glucose 6-phosphate. The glucose 6-phosphate can be used in the glycogenesis pathway or can be converted to pyruvate through glycolysis. Pyruvate kinase (PK) catalyses the last step of glycolysis to produce pyruvate. Pyruvate is subsequently used in either the lipogenesis pathway or in the Krebs cycle to provide energy (Fig. 1.3 in Chapter 1). Higher dietary carbohydrate increased the PK activity in many fish (reviewed by Enes et al., 2009). Metabolic pathways involved with the catabolism of dietary carbohydrate as energy in fish are significant to improve protein utilisation. In carnivorous fish, protein utilisation was improved significantly at higher dietary carbohydrate levels associated with increased glycolytic enzyme such as GK and PK activities (Enes et al., 2006a). For dietary carbohydrate to provide energy, the glucose carbon must enter the Krebs cycle as pyruvate, convert to acetyl-coA and then be completely oxidised to carbon dioxide

(Rawles et al., 2008). Alternatively, the excess carbohydrate is catabolised as storage of glycogen (glycogenesis) or can be catabolised through the hexose monophosphate shunt to provide NADPH for subsequent production of lipid (lipogenesis; Fig. 1.3 in Chapter 1). When dietary carbohydrate is insufficient to meet the energy requirement, glucose is synthesised from non-carbohydrate precursors such as lactate, glycerol, or α -ketoacids or from protein catabolism through *de novo* synthesis of glucose (gluconeogenesis; Fig. 1.3 in Chapter 1).

In some fish, excess carbohydrate can be converted to lipid (lipogenesis) through the hexose monophosphate shunt (Fig. 1.3 in Chapter 1), also called the pentose phosphate pathway. It is the most important alternative pathway, catalysed by action of glucose 6-phosphate dehydrogenase (G6PDH), lactonase, 6-phosphogluconodehydrogenase (6PGDH) and phosphopentoisomerase. The activity of glucose 6-phosphate dehydrogenase (G6PDH) was higher in rainbow trout (Hilton and Atkinson, 1982) and spotted babylon (Zhang et al., 2009) fed higher dietary starch level. Higher activity of G6PDH and 6PGDH indicated that the pentose phosphate pathway was active to metabolise the excess glucose to provide NADPH for subsequent lipogenesis (Metón et al., 2003).

Protein efficiency is decreased in fish fed lower dietary energy where dietary protein is catabolised to supply energy rather than being used for growth. Protein degradation describes the deamination of amino acids obtained either from dietary protein or muscle protein (Kumar et al., 2009). Protein degradation occurs through gluconeogenesis involving deamination of amino acids catalysed by glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT) or asparate aminotransferase (ASAT) (Fig. 1.3 in Chapter 1). A protein-rich diet in gilthead sea bream, *Sparus aurata* increased the activity of ALAT and GDH indicated that excess dietary protein was used for energy purposes or for glucose or lipid synthesis (Enes et al., 2008a). When gilthead sea bream were fed with high levels dietary carbohydrate, ALAT and GDH activity were decreased and subsequently protein catabolism decreased (Enes et al., 2008a).

Carbohydrate rich diets increased glycogen content in livers of European whitefish, *Coregonus lavaretus* (Vielma et al., 2003), rainbow trout (Suarez et al., 2002; del sol

Novoa et al., 2004), Atlantic halibut, *Hippoglossus hippoglossus* (Hatlen et al., 2005), sunshine bass, *Morone chrysops* ♀ x *M. saxatilis* ♂ (Hutchins et al., 1998), European sea bass, *Dicentrarchus labrax* (Moreira et al., 2008) and perch, *Perca fluviatilis* (Borrebaek et al., 2003). Some omnivorous fish fed diets rich in carbohydrate showed hypertrophy in hepatocytes and vacuolation in the liver (Mohapatra et al., 2003; Kumar et al., 2005; Yengkokpam et al., 2005). Higher dietary carbohydrate caused histological damage in the liver of carnivorous fish which was related to ruptured hepatocyte membranes, swelling and vacuolation of the hepatocytes (Cheng et al., 2007). Necrotic hepatocytes in the liver of piscivorous largemouth bass, *Micropterus salmoides* was attributed to progressive accumulation of glycogen in liver (Goodwin et al., 2002).

Temperature affects the carbohydrate utilisation and hepatic enzyme activity. High temperature enhanced liver glycolytic, gluconeogenic and lipogenic capacities of European sea bass and gilthead sea bream indicating that those two species utilised carbohydrate efficiently at high temperature (Enes et al., 2006b; Enes et al., 2008c). Glucokinase enzyme activities of gilthead sea bream was higher at 25°C than 18°C (Enes et al., 2008b). Similarly, glycolytic enzyme activity of European sea bass and gilthead sea bream was higher at high temperature, where as FBPase, G6PDH and GDH was not affected by temperature (Couto et al., 2008; Moreira et al., 2008). Starch is better utilised than glucose by grouper, *Epinephelus malabaricus* when reared at 23°C due to the increased activity of GK (Shiau and Lin, 2002). Rainbow trout could utilise more carbohydrate at 15°C than 10°C (Hilton et al., 1982). Again, this fish was able to utilise more dietary carbohydrate for energy at 18°C in comparison to 8°C (Brauge et al., 1995).

Like other carnivorous and salmonid fish, brook trout in their natural environment grow on food especially devoid of carbohydrate (Scott and Crossman, 1973) and they are more adapted to metabolise protein and lipid than carbohydrate (Wilson, 1994; Moreira et al., 2008). Although the maximal tolerable limit of carbohydrate in rainbow trout was 14% (Hilton et al., 1982), studies for other salmonids recommend a maximum of 20% of diet (Pieper and Pfeffer, 1980; Spannhof and Plantikow, 1983; Wilson, 1994). As yet, there are no studies available for salmonids examining the metabolic adaptability to increased level of gelatinised carbohydrate in relation to water temperature. There are

few studies available about the effect of water temperature and carbohydrate levels on the hepatic enzyme adaptation in European sea bass (Couto et al., 2008) and gilthead sea bream (Moreira et al., 2008). Studies on hepatic enzymatic adaptation in brook trout fed gelatinised carbohydrate in relation to temperature may discover the potential to increase the use of gelatinised carbohydrate in other carnivorous fish including salmonids. The aim of this study is, therefore, to evaluate the effect of twelve levels of dietary gelatinised carbohydrate (0.1 to 28%) on the key hepatic enzyme activities and liver histology of brook trout at two temperatures (15°C and 19°C).

3.3. Materials and Methods

3.3.1. General methods

The general methods regarding fish handling, diet preparation and feeding, water quality monitoring and sampling used in this chapter are described in Chapter 2 (Sections 2.3.1 to 2.3.3). Briefly, this chapter describes the activity of key hepatic enzymes (PK, GDH and G6PDH) and histological features of the liver in brook trout fed twelve diets with low to high levels of carbohydrate (0.1 to 28.4%) balanced by increasing protein levels (Table 3.1). The experiment was conducted in two freshwater recirculation systems at the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), University of Tasmania, Launceston, Australia. Each system had twelve 300 L tanks. One system was maintained at 15°C and the other at 19°C. Each of twelve diets was allocated to one tank of fish, in both temperature systems. During the experiment fish were fed the experimental diet at a uniform ration of 2% of body weight which was equally divided in two feeds (9:30 and 15:30) daily. At the end of the experiment individual fish were weighed (g) and length measured (cm) (chapter 2). Five fish fed three different levels of dietary carbohydrate, 28.4% (36% protein), 10% (50% protein) and 0.1% (58% protein) were sacrificed (Aqui-S, 400 ppm, AQUI-S New Zealand Ltd.) and liver samples were taken for histology and enzyme analysis.

Table 3.1. The ingredient and chemical composition of experimental feeds (g·kg⁻¹)

Ingredient Inclusion	P36	P38	P40	P42	P44	P46	P48	P50	P52	P54	P56	P58
Fish meal	395	424	453	482	511	541	570	599	628	658	687	716
Wheat gluten	100	100	100	100	100	100	100	100	100	100	100	100
Fish oil	154	150	147	144	140	137	133	130	126	123	119	116
Pregelatinised maize starch	284	259	233	207	182	155	130	104	79	52	27	1
Vitamins ^a	15	15	15	15	15	15	15	15	15	15	15	15
Minerals ^b	15	15	15	15	15	15	15	15	15	15	15	15
Binder CMC	10	10	10	10	10	10	10	10	10	10	10	10
Stay C	15	15	15	15	15	15	15	15	15	15	15	15
Choline chloride	1	1	1	1	1	1	1	1	1	1	1	1
Monobasic calcium phosphate	10	10	10	10	10	10	10	10	10	10	10	10
Ytterbium oxide	1	1	1	1	1	1	1	1	1	1	1	1
<i>Chemical composition (dry matter)</i>												
Dry matter	926.5	921.1	919.9	927.1	921.0	913.6	924.1	917.8	915.4	915.5	914.3	923.5
Crude protein	377.7	385.8	411.1	430.7	446.5	461.8	490.7	502.4	532.4	555.6	572.2	601.0
Crude lipid	231.4	233.5	237.1	240.5	238.3	243.7	245.1	241.4	238.7	233.9	235.4	242.5
Ash	105.2	106.4	107.5	109.4	110.7	116.0	117.0	119.3	124.7	131.1	144.7	145.8
NFE ^c	285.7	274.3	244.4	219.4	204.6	178.5	147.2	136.9	104.2	79.4	47.7	10.7
Gross energy (MJ·kg ⁻¹)	22.72	22.66	23.31	23.00	23.22	23.02	22.45	22.63	22.81	23.26	22.35	22.70

^a Vitamin premix (mg·kg⁻¹ of mixture) = Vitamin A acetate (ICN), 7.50; Vitamin D3 powder (ICN), 9.00; Rovimix E50, 150.00; Menadone sodium bisulphate, 3.00; Riboflavin, 6.00; Calcium D-pantothenate, 32.68; Nicotinic Acid, 15.00; Vitamin B12, 0.015; d-Biotin, 0.225; Folic Acid, 1.50; Thiamin HCL, 1.68; Pyridoxine HCL, 5.49; Myo-Inositol, 450.00; α -cellulose, 817.91; Stay-C, 150.00.

^b Mineral premix (mg·kg⁻¹ of mixture) = CuSO₄ 5H₂O (cupric sulphate), 35.37; FeSO₄ 7H₂O (ferrous sulphate), 544.65; MnSO₄ H₂O (manganese sulphate), 92.28; Na₂SeO₃ (sodium selenate), 0.99; ZnSO₄ 7H₂O (zinc sulphate), 197.91; KI (potassium iodide), 2.16; CoSO₄ 7H₂O (cobalt sulphate), 14.31; α -cellulose, 612.33.

^c NFE (dry matter) = 100 – (Crude protein% + Crude lipid % + Ash%)

3.3.2. General histology

The liver was bisected and a 4 mm slice from the middle of liver was taken and fixed in 10% neutral buffered formalin (pH 7.0). Liver samples were then embedded in paraffin, sectioned (5 μ m) and stained with haematoxylin and eosin (Roberts, 1989). To assess the presence of glycogen in the liver, slides from fish fed 0.1% and 28.4% level of carbohydrate at both temperatures were stained with Periodic acid-schiff (PAS) reagent (Bancroft et al., 1996). One sample slide from each group was used as a positive control for glycogen and was treated with salivary amylase, and all negative controls with distilled water. Slides were incubated for 15 min at 37°C, rinsed with distilled water and dried before staining with PAS reagent.

3.3.3. Enzyme activity analysis

The remaining liver sample was immediately frozen in liquid nitrogen and stored at -80°C for hepatic enzyme activity analysis. Due to availability of enzyme kit and chemical every alternative treatment are analysed for enzyme. Sample of seven treatments for each temperature were analysed for PK and G6PDH. For GDH, sample of six treatments for both temperatures were analysed. Liver samples (n=5) were pooled per tank for each dietary treatment for analysis. Assay kits for PK (Pyruvate Kinase Assay Kit, K709-100), GDH (Glutamate Dehydrogenase Assay Kit, K729-100) and G6PDH (Glucose 6-Phosphate Dehydrogenase Assay Kit, K757-100); BioVision Research Products, 980 Linda Vista Avenue, Mountain View, CA 94043, USA) were used to analyse the activity of hepatic PK, GDH and G6PDH.

For PK activity, liver samples (approx. 50 mg) were homogenised with 4 volumes of kit assay buffer (part number K709-100-1) with an electric homogeniser (Ystral, D-79282, Ballrechten, Dottingen, Germany) and centrifuged (13000 g for 10 min at 4°C) to obtain a clear extract. The supernatant was taken as the test sample extract. A reaction mix was prepared using supplied assay buffer, substrate mix, enzyme mix and OxiRed™ probe, while no substrate mix was added for the blank mixtures. Optical density was measured at 570 nm using 96-well plate reader (Tecan Spectra Rainbow Microplate Reader, Tecan Group Ltd, Switzerland) over two times, at immediately after adding reaction mix and after incubating for 30 min at 25°C.

For GDH activity, liver samples (approx. 50 mg) were homogenised with 4 volumes of kit assay buffer (part number K729-100-1) with an electric homogeniser and centrifuged (13000 g for 10 min at 4°C) to obtain a clear extract. The supernatant was taken as the test sample extract. A reaction mix was prepared using supplied assay buffer, GDH developer and glutamate. The test sample extract with reaction mixture was incubated for 3 min at 37°C and optical density was measured at 450 nm using 96-well plate reader at first time. Optical density was measured at second time after incubating at 37°C for 30 min.

For G6PDH activity, liver samples (approx. 100 mg) were homogenised with equal volumes of ice cold phosphate buffer saline (PBS, pH 7.4, Sigma) with an electric homogeniser and centrifuged (13000 g for 10 min at 4°C) to obtain a clear extract. The supernatant was taken as the test sample extract. A reaction mix was prepared using supplied assay buffer, G6PDH developer and G6PDH substrate. Optical density at 450 nm using 96-well plate reader was measured over two times, at immediately after adding reaction mix and after incubating for 30 min at 37°C (Chen et al., 2011).

3.3.4. Statistical analysis

Data were presented as mean \pm standard error. Effects of dietary carbohydrate on hepatosomatic index and enzyme activity were analysed by polynomial regression. The difference between two temperatures were analysed by ANCOVA. Data were analysed using SPSS software package version 19.0 (SPSS Inc, IBM, IL, USA). Piecewise regression model was performed to determine the break point of dietary carbohydrate level on hepatic enzymatic activity (Ryan and Porth, 2007). The probability level of 0.05 or less was considered as significant for rejection of the null hypothesis.

3.4. Results

3.4.1. Hepatosomatic index (HSI)

Hepatosomatic index increased from 1.25 to 5.03 at 15°C and 1.50 to 3.80 at 19°C with increasing dietary carbohydrate (Fig. 3.1). A significant quadratic relationship between the HSI of fish and carbohydrate (CHO) level was found at both temperatures and can

be expressed by following equations $HSI (15^{\circ}C) = 0.005 \pm 0.001(\%CHO)^2 - 0.022 \pm 0.043(\%CHO) + 1.468 \pm 0.264$ ($R^2=0.93$, $F_{2,9}=58.399$, $P<0.001$) and $HSI (19^{\circ}C) = 0.002 \pm 0.001(\%CHO)^2 + 0.01 \pm 0.028(\%CHO) + 1.551 \pm 0.172$ ($R^2=0.93$, $F_{2,9}=58.338$, $P<0.001$). The HSI of brook trout fed experimental feeds was not significantly affected by temperatures ($F_{1,11}=1.378$, $P=0.265$) and it was $HSI = 0.004 \pm 0.001(\%CHO)^2 - 0.006 \pm 0.031(\%CHO) + 1.509 \pm 0.189$ ($R^2=0.88$, $F_{2,21}=78.035$, $P<0.001$).

3.4.2. Histological studies

Liver tissue of brook trout fed a diet containing 0.1% carbohydrate (58% level of protein) had normal cellular with dense uniformly sized hepatocytes (Fig. 3.2, plate A & B). Swelling and vacuolation in hepatocytes was observed in fish which were fed dietary carbohydrate level of 10.0% (50% level of protein) (Fig. 3.2, plate C & D). Hepatocytes from fish fed dietary carbohydrate level of containing 28.4% (36% level of protein) appeared swollen with moderate vacuolation (Fig. 3.2, plate E & F). The vacuolated hepatocytes were PAS positive (glycogen granules: pink coloured) in negative control (Fig. 3.3, B & D). The positive control slides containing brook trout liver were PAS negative after digestion in salivary amylase (Fig. 3.3, A, C). There were no distinct differences found between positive and negative control slides (Fig. 3.3 E & F, G & H) containing liver of brook trout fed 0.1% of gelatinised carbohydrate.

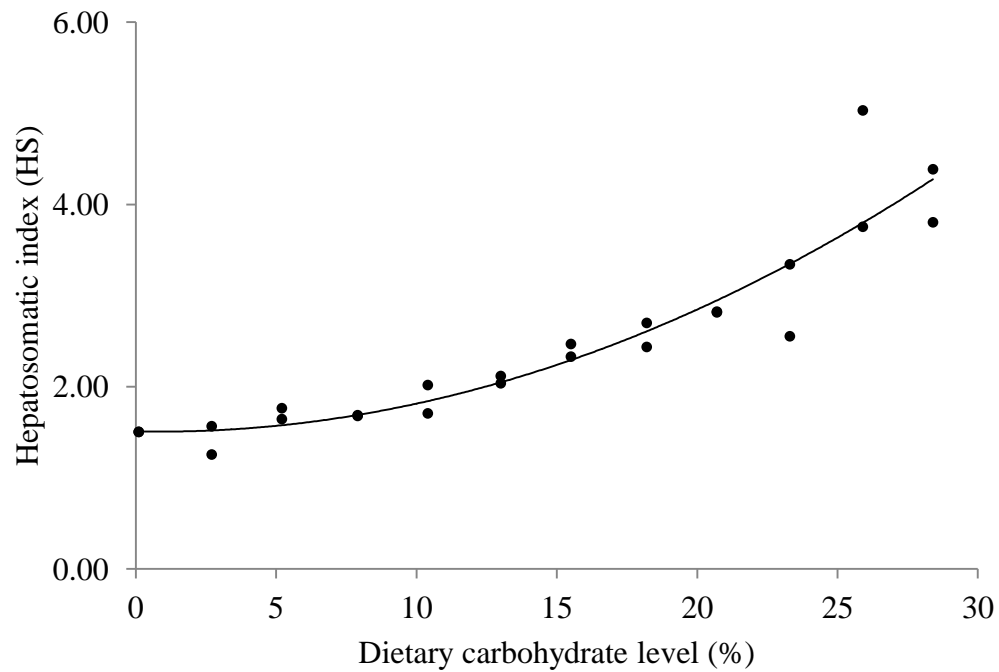


Figure 3.1. Hepatosomatic index (HSI) of brook trout, *Salvelinus fontinalis* fed different levels of gelatinised maize starch balanced by protein. The relationship can be expressed as $HSI = 0.004 \pm 0.001(\%CHO)^2 - 0.006 \pm 0.031(\%CHO) + 1.509 \pm 0.189$ ($R^2=0.88$, $F_{2,21}=78.035$, $P<0.001$).

Figure 3.2. Liver histology from brook trout, *Salvelinus fontinalis* fed different diets containing varying level of protein and carbohydrate at 15°C and 19°C (Haematoxylin and eosin). Fig. A (15°C) & B (19°C): Diet containing 0.1% carbohydrate and 58% protein; hepatocytes appeared normal cellular structure. Fig. C (15°C) & D (19°C): Diet containing 10% carbohydrate and 50% protein; Hepatocytes appeared swollen with vacuolation (scale bar = 50 µm). Fig. E (15°C) and F (19°C): Diet containing 28.4% carbohydrate and 36% protein; hepatocytes appeared swollen with moderate vacuolation (scale bar = 50 µm) (scale bar = 50 µm).

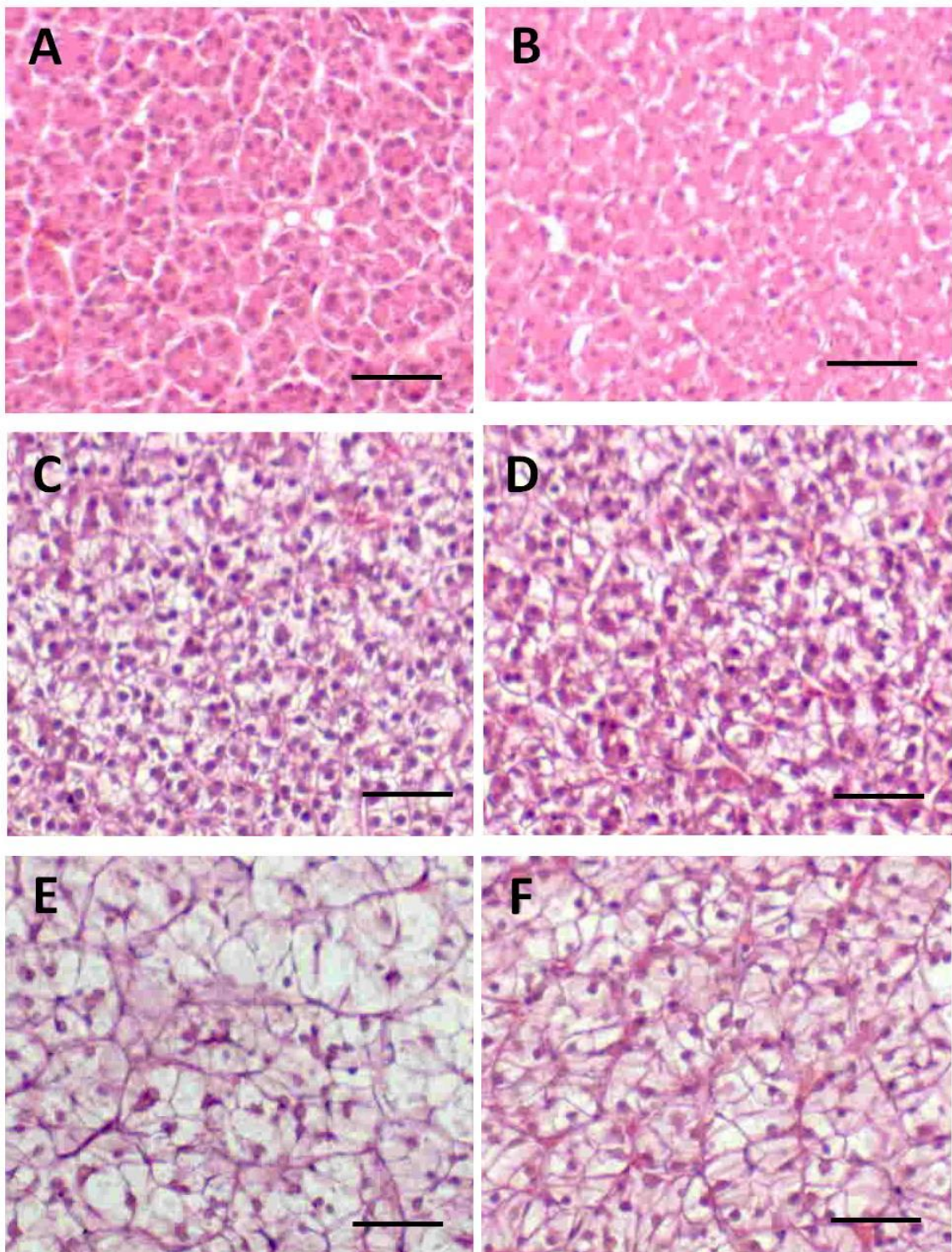
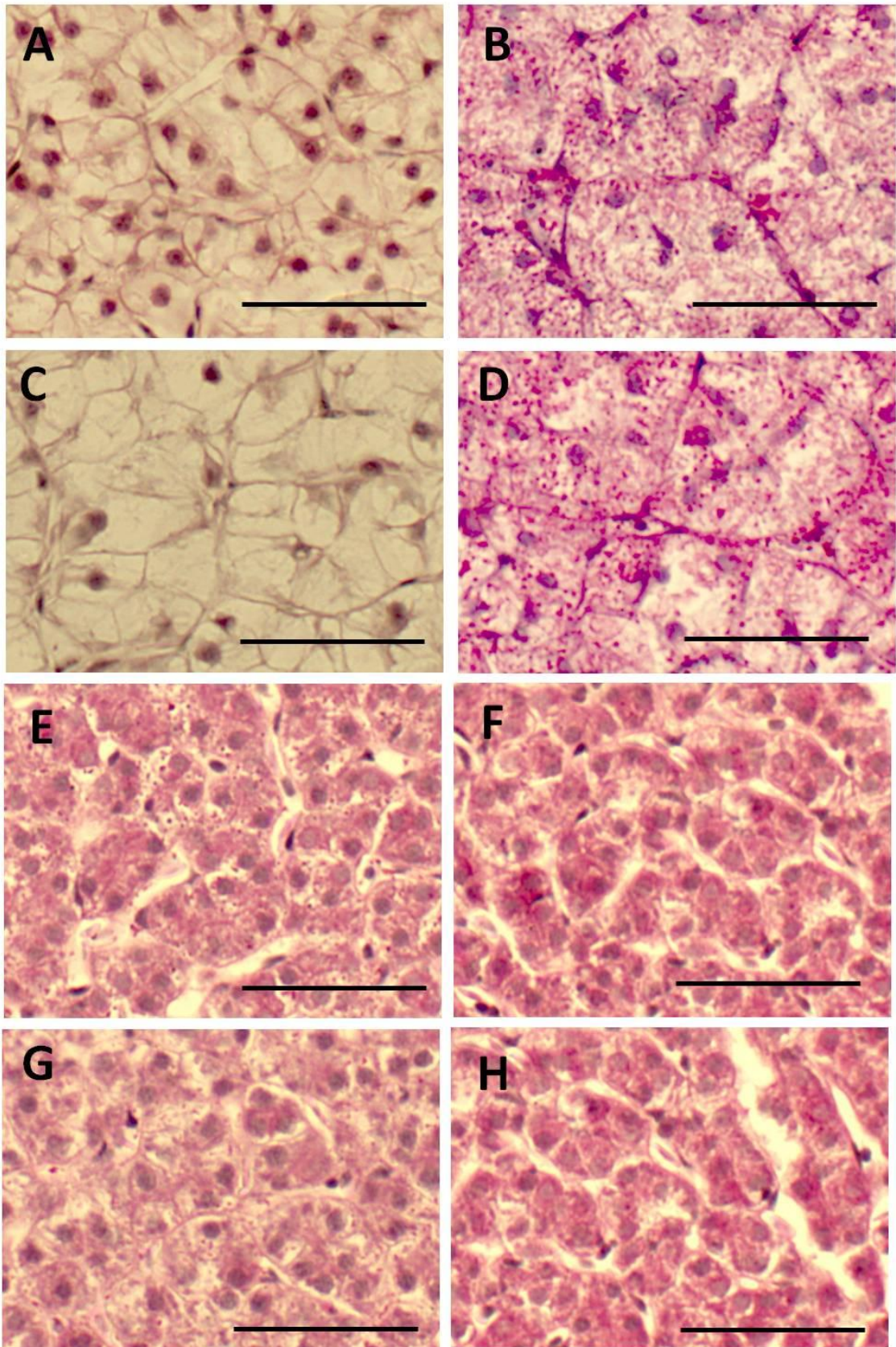


Figure 3.3. Liver histology with histochemical staining (Periodic acid-schiff, PAS) for glycogen in brook trout, *Salvelinus fontinalis*, fed different diets containing varying level of carbohydrate (scale bar = 50 μ m) at 15°C and 19°C. The vacuolated hepatocytes were PAS positive (glycogen granules: pink coloured) in negative control (Fig. B and D). The positive control slides containing brook trout liver were PAS negative after digestion in salivary amylase (Fig. A and C). Fig. A, B, C and D: Fish fed diets containing 28.4% level of carbohydrate. Fig. A and B: fish reared at 15°C; Fig. C and D: fish reared at 19°C. There were no distinct differences found between positive control (Fig. E and G) and negative control (Fig F and H) slides containing liver of brook trout fed 0.1% of gelatinised carbohydrate. Fig. E and F: fish reared at 15°C; Fig. G and H: Fish reared at 19°C.



3.4.3. Hepatic enzyme activity

Hepatic PK activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ liver) increased from 1.58 to 36.05 at 15°C and 12.46 to 62.39 at 19°C with increasing dietary carbohydrate (Fig. 3.4). The hepatic PK activity of brook trout was significantly affected by dietary carbohydrate level at both temperatures ($R^2=0.83$, $F_{2,4}=10.028$, $P=0.028$ at 15°C and $R^2=0.95$, $F_{2,4}=34.393$, $P=0.003$ at 19°C). There were no clear breakpoints of dietary carbohydrate level on PK activity observed at both temperatures when the data were modelled by piecewise regression (not shown in figure). Pyruvate kinase activity was higher in 19°C than 15°C ($F_{1,6}=9.802$, $P=0.020$).

Hepatic G6PDH activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ liver) were neither affected by dietary carbohydrate ($R^2=0.63$, $F_{2,4}=3.425$, $P=0.136$ at 15°C; $R^2=0.11$, $F_{2,4}=0.237$, $P=0.799$ at 19°C) or by temperatures ($F_{1,6}=2.136$, $P=0.194$) (Fig. 3.5). The average hepatic G6PDH activity (mean \pm SE) was 73.03 ± 2.31 at 15°C and 76.57 ± 1.41 at 19°C. There were no clear breakpoints of dietary carbohydrate level on G6PDH activity observed at both temperatures when the data were modelled by piecewise regression (not shown in figure).

Hepatic GDH activity was significantly higher at 19°C than 15°C ($F_{1,5}=34.662$, $P=0.002$) (Fig. 3.6). Hepatic GDH activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ liver) of brook trout was significantly affected by dietary carbohydrate and GDH activity was peaked at 24.94 (15°C) and 27.77 (19°C) in the liver of brook trout and fed diet containing 13% carbohydrate ($R^2=0.96$, $n=6$, $P<0.001$ at 19°C and $R^2=0.91$, $n=6$, $P=0.003$ at 15°C) (Fig. 3.6).

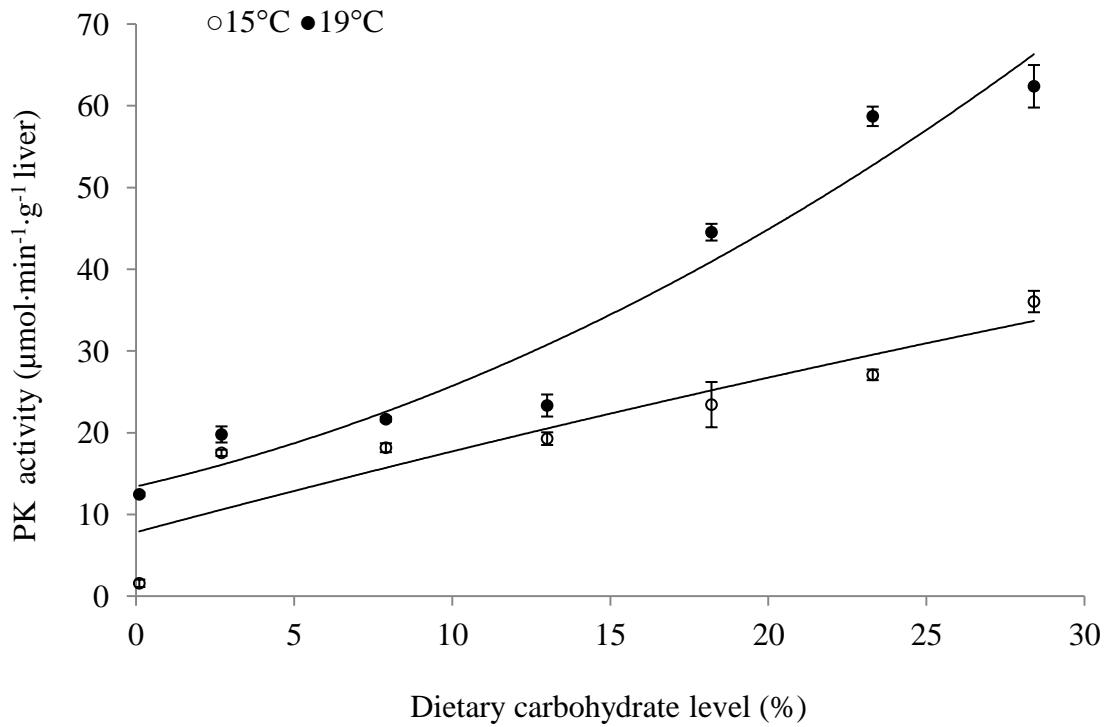


Figure 3.4. Hepatic pyruvate kinase activity of brook trout, *Salvelinus fontinalis* fed different levels of gelatinised maize starch balanced by protein. At 15°C, the relationship can be expressed as $\text{PK activity} = -0.004 \pm 0.026(\% \text{CHO})^2 + 1.031 \pm 0.749(\% \text{CHO}) + 7.831 \pm 4.232$ ($R^2=0.83$, $F_{2,4}=10.028$, $P=0.028$) and at 19°C it can be expressed as $\text{PK activity} = 0.034 \pm 0.028(\% \text{CHO})^2 + 0.887 \pm 0.826(\% \text{CHO}) + 13.423 \pm 4.4667$ ($R^2=0.95$, $F_{2,4}=34.393$, $P=0.003$).

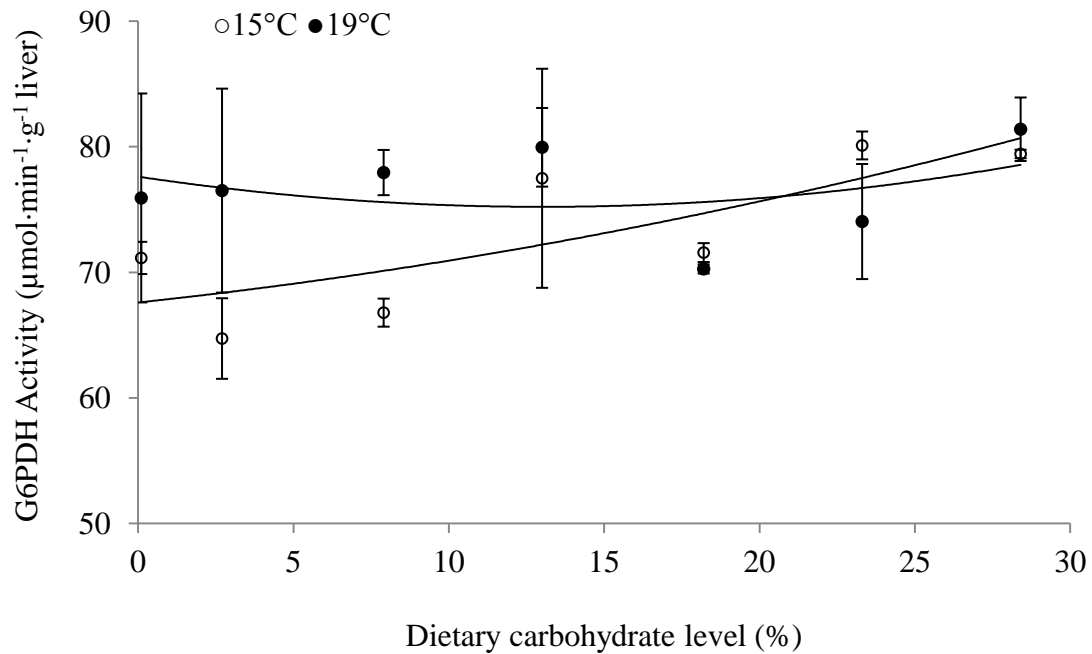


Figure 3.5. Hepatic glucose 6-phosphate dehydrogenase activity of brook trout, *Salvelinus fontinalis* fed different level of gelatinised maize starch balanced by protein. At 15°C, the relationship can be expressed as $G6PDH \text{ activity} = 0.007 \pm 0.022(\%CHO)^2 + 0.264 \pm 0.647(\%CHO) + 67.596 \pm 3.654$ ($R^2=0.63$, $F_{2,4}=3.425$, $P=0.136$) and at 19°C it can be expressed as $G6PDH \text{ activity} = 0.014 \pm 0.021(\%CHO)^2 - 0.369 \pm 0.613(\%CHO) + 77.618 \pm 3.463$ ($R^2=0.11$, $F_{2,4}=0.237$, $P=0.799$).

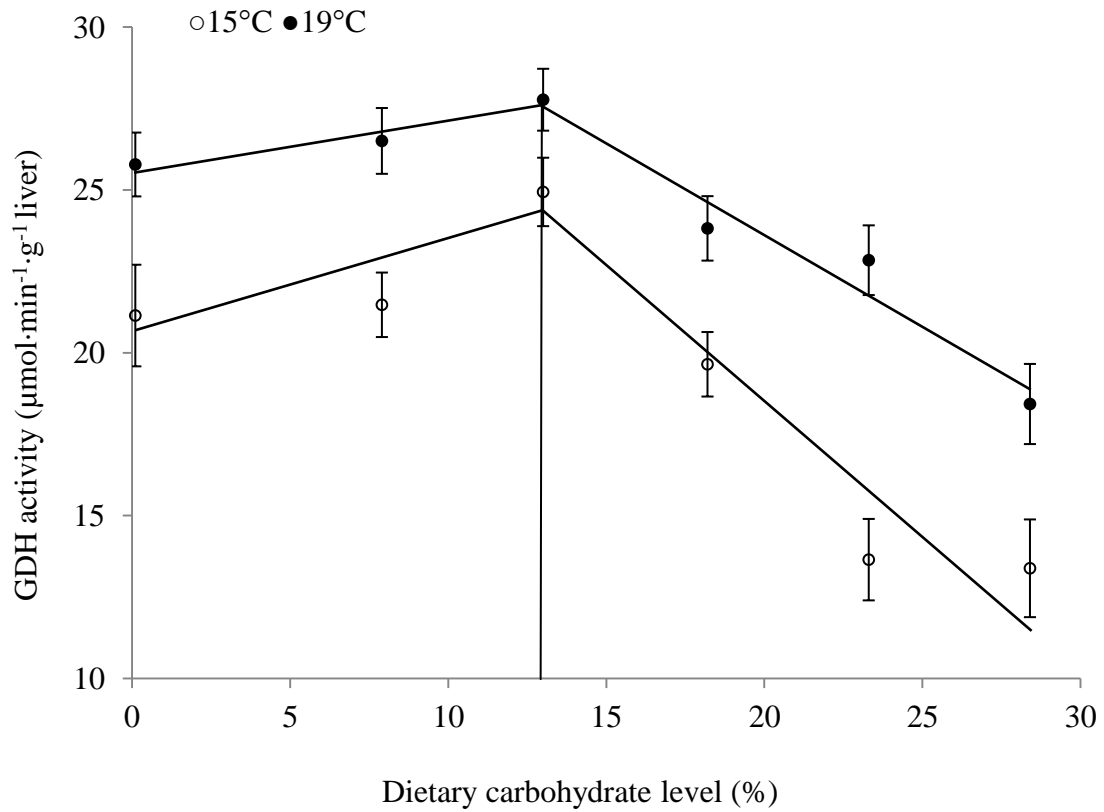


Figure 3.6. Hepatic glutamate dehydrogenase (GDH) activity of brook trout, *Salvelinus fontinalis* fed different level of gelatinised maize starch balanced by protein. By using piecewise regression model, the GDH activity was peaked to 24.94 at 15°C and to 27.77 at 19°C when fish fed diet containing 13% carbohydrate ($R^2=0.96$, $n=6$, $P<0.001$ at 19°C and $R^2=0.91$, $n=6$, $P=0.003$ at 15°C).

3.5. Discussion

Carnivorous fish like brook trout are generally thought to be less capable of utilising dietary carbohydrate. The qualitative features of liver and hepatic enzymatic regulation of brook trout fed dietary carbohydrate were examined to determine the potential of using gelatinised carbohydrate in the diet of brook trout. The observed positive relationship between HSI and dietary carbohydrate level was most likely related to increased energy storage (Daniels and Robinson, 1986; Hidalgo and Alliot, 1988; Hemre et al., 1989; Yang et al., 2003).

Liver samples analysed for histology in this study revealed that vacuolation became more obvious in fish fed 28%, compared to 0.1% and 10% gelatinised carbohydrate. The degree of vacuolation in hepatocytes was positively related with increasing dietary carbohydrate from 13% to 25% in largemouth bass (Amoah et al., 2008). Vacuolated hepatocytes was found in fish fed high carbohydrate as results of glycogen deposition in liver (Mohapatra et al., 2003; Kumar et al., 2005; Yengkokpam et al., 2005; Moreira et al., 2008). Glycogen is the form of reserve energy, used to meet sudden energy requirements, when depleted, body lipid is catabolised for energy; if both glycogen and lipid are depleted muscle protein is degraded for energy (Pérez-Jiménez et al., 2007). Thus, brook trout liver glycogen store reduce protein degradation for energy (Pérez-Jiménez et al., 2007).

The PK activity was $5.43 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ liver in rainbow trout (reared at 15°C) fed diet 3.2% crude starch and 10.6% sugar (Hilton and Atkinson, 1982). The PK activity of rainbow trout (reared at 15°C) increased from 21.2 to $52.7 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ liver, when fish fed 10% carbohydrate with 60% protein and 56% carbohydrate with 20% protein, respectively. The highest PK activity in the liver of brook trout was 36.05 (reared at 15°C) and 62.39 (reared at 19°C) when fish fed 28% gelatinised carbohydrate indicated that possible metabolic adaptation for gelatinised carbohydrate particularly at high temperature. However, the PK activity increased with higher levels of dietary carbohydrate indicating that brook trout actively regulated PK activity to catabolise carbohydrate for either energy production or lipid synthesis. The similar trend was found in rainbow trout where higher PK activity with high carbohydrate diets was

occurring indicated increasing energy production from carbohydrate metabolism (Walton, 1986). Higher carbohydrate increased the energy production which improved the protein efficiency in common carp, *Cyprinus carpio* (Capilla et al., 2004). The higher PK activity was related to protein sparing effect of carbohydrate and also contributed to explain the increased liver glycogen levels and HSI in gilthead sea bream fed higher carbohydrate diets (Couto et al., 2008). Higher protein efficiency was reported in brook trout fed higher level of carbohydrate (chapter 2).

Brook trout reared at 19°C showed higher PK activity than 15°C indicated that glycolytic process of carbohydrate was higher at elevated temperature. This result was consistent with other carnivorous fish such as gilthead sea bream and European sea bass, where warmer temperatures increased the glycolytic process (Couto et al., 2008; Enes et al., 2008b; Enes et al., 2008c). Although there was no clear optimum found, compared to 15°C, the rate of increasing PK activity at 19°C was higher in brook trout liver, fed more than 13% of gelatinised carbohydrate. However, according to this study, it can be suggested that brook trout have higher glucose metabolism potential at high temperature and dietary gelatinised carbohydrate can be used as potential sources of energy at high temperatures which are frequently encountered during the summer months.

High gelatinised carbohydrate reduced the GDH activity in the liver of brook trout and reduced protein degradation suggesting that gelatinised carbohydrate may lead to increased protein synthesis. The GDH activity was peaked when fish fed 13% gelatinised carbohydrate at both temperatures indicated that brook trout diet should contain more than that level to reduce muscle protein catabolism. It was found in common carp that higher levels of carbohydrate increased glucose utilisation and reduced protein degradation therefore resulting in increased protein retention (Capilla et al., 2004). Thus, it is crucial to supply available alternative energy rather than increasing protein to achieve better protein utilisation as growth. Higher dietary starch level decreased GDH activities in carnivorous fish including gilthead sea bream and European sea bass, suggesting that protein catabolism decreased with the inclusion of starch in the diet (Enes et al., 2006a; Fernández et al., 2007; Enes et al., 2008a).

In fish, NADPH obtained from G6PDH activity is required for lipogenesis. There was no clear effect of either diet or temperature on G6PDH, suggesting that hexose monophosphate shunt was not stimulated by dietary carbohydrate level or by temperature. Similarly, G6PDH activity was not regulated by dietary starch level in European sea bass (Dias et al., 1998; Enes et al., 2006a) or by water temperature in Senegalese sole, *Solea senegalensis* (Guerreiro et al., 2012a; Guerreiro et al., 2012b). In brook trout the whole body lipid content and productive lipid value were increased with higher gelatinised carbohydrate (Chapter 2) and may suggest increased *de novo* lipogenesis. However, the lack of upregulation of G6PDH, together with increased body lipid and increased activity of PK with increasing dietary carbohydrate may imply that alternative pathways involved to synthesise fatty acid from acetyl- CoA might be active in brook trout instead of the hexose monophosphate shunt (Fig. 1.3 in Chapter 1). For instance in rainbow trout, the extra supply of acetyl-CoA was obtained from hepatic glycolysis (Hilton and Atkinson, 1982). Metabolic pathways involved to synthesise lipid from fructose 1, 6-biphosphate via glucose 3-phosphate could be possible (Fig. 1.3 in Chapter 1), need to be investigated. These findings are important to understand the potential for effective use of gelatinised carbohydrate, which may either lead to lipogenesis, reduceing the need for dietary lipid or lipid sparing. Carbohydrate diets tend to accumulate whole-body lipid in fish, increase the efficiency of lipid biosynthesis from dietary lipid and decrease the contribution of lipid to oxidative metabolism (Hemre et al., 2002). It might be possible to enhance the hexose monophosphate shunt to generate NADPH required for fatty acids synthesis in trout fed low lipid with high carbohydrate diets (Hilton and Atkinson, 1982).

In conclusion, this study revealed that increasing water temperature increased the activity of glycolytic enzyme (PK) indicating that brook trout are more capable of using gelatinised maize starch at higher temperatures. As well as at both temperatures (15°C or 19°C) higher levels of gelatinised starch increased the glycolytic enyme (PK) activity and also reduce protein catabolic enzyme (GDH) activity. Thus, gelatinised maize starch can be used as a good source of non protein energy in the diet of brook trout. At least 13% gelatinised carbohydrate should be added in the diet to reduce protein catabolism.

3.6. References

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CHAPTER 4

EFFECT OF TEMPERATURE AND VARYING LEVEL OF CARBOHYDRATE AND
LIPID ON GROWTH, FEED EFFICIENCY AND NUTRIENT DIGESTIBILITY OF BROOK
TROUT, *SALVELINUS FONTINALIS* (MITCHILL, 1814)

4.1. Abstract

The effect of gelatinised carbohydrate level on growth performance, nutrient utilisation and digestibility of brook trout, *Salvelinus fontinalis* was evaluated at two temperatures (15°C and 19°C). Four iso-nitrogenous (44% protein) and iso-energetic (22 MJ·kg⁻¹) diet were formulated with increasing carbohydrate level (18-26%) balanced by decreasing lipid level (17-13%). Fish were fed 2% body weight ration daily. Growth rate was higher and feed utilisation was more efficient at 15°C than 19°C ($P<0.001$). However, diets had no effect on growth and feed utilisation. Apparent digestibility (%) of nutrient and energy were significantly higher at 15°C than 19°C ($P<0.001$). Improved apparent digestibility of dry matter (AD_{DM}), gross energy (AD_{GE}) and energy from carbohydrate (AD_{CHO-E}) with 26% carbohydrate at both temperatures indicated that brook trout can compensate their energy requirement from carbohydrate instead of lipid. Levels of gelatinised starch had positive effect on the activity of α -amylase, which was higher at 15°C. Across the levels of dietary carbohydrate tested, there were no pathological changes to liver or intestine histology. At 15°C and elevated summer water temperatures of 19°C, commonly experienced in Australian aquaculture, high carbohydrate inclusion replaced fish oil with no detriment to health, nutrient digestibility or growth of brook trout.

4.2. Introduction

The most efficient diets present a surplus amount of non-protein energy sources (carbohydrate or lipid) which can be metabolised to meet general energy requirements, leaving an organism to direct the maximum level of available dietary protein into growth (Johnston et al., 2003). It is generally believed that carnivorous fish have limited capacity to utilise carbohydrate (Wilson, 1994; Stone, 2003). Higher digestibility of gelatinised starch has been described in many fish including carnivorous fish species such as gilthead sea bream, *Sparus aurata*, European sea bass, *Dicentrarchus labrax*, rainbow trout, *Oncorhynchus mykiss* (Bergot, 1993; Peres and Oliva-Teles, 2002; Stone, 2003; Alexander et al., 2011; Couto et al., 2012). The effect of different carbohydrate levels on growth, protein efficiency and nutrient digestibility has been studied in several carnivorous fish and showed that an increase in dietary carbohydrate did not affect the growth performances (Dias et al., 2004; Moreira et al., 2008; Guerreiro et al., 2012b), feed efficiency (Dias et al., 2004; Guerreiro et al., 2012b), starch digestibility (Couto et al., 2012), protein efficiency and protein digestibility (Moreira et al., 2008). Due to the lack of nutritional information on brook trout, *Salvelinus fontinalis* (Jobling et al., 2010), carbohydrate utilisation and effects of energy source on liver and gut have not been established. However, it has been determined in rainbow trout that dietary protein utilised for energy could be replaced considerably by carbohydrate resulting in the dietary protein content being reduced from 68% to 34% and replaced with increasing amounts of carbohydrate which in turn significantly improved protein efficiency (Pieper and Pfeffer, 1980a). Similar results were found in brook trout where increasing carbohydrate improved protein efficiency (Chapter 2). An iso-lipidic and iso-energetic diet, achieved by replacing dietary protein content with carbohydrate showed no difference in the energy efficiency and positive effect on protein efficiency (Krogdahl et al., 2004). Therefore, further information is needed to replace lipid by carbohydrate and to determine the proper balance of carbohydrate and lipid to obtain the best growth and feed efficiency.

Water temperature is the most important abiotic factor affecting the growth of fish (Brett, 1979). Fish required more metabolic energy at high temperature, to compensate for the increased energy demand which peaked prior to the upper limit for thermal

tolerance (Jobling, 1994). The optimum temperature for growth is slightly lower than that for feed intake and the difference between the feed intake and metabolic rate indicates the energy available for growth (Jobling, 1997; Katersky and Carter, 2005). For any given size of ration, the scope for growth (energy availability) decreases as temperature is increased (Jobling, 1994). Maximum feed conversion efficiency occurs at a temperature that was slightly lower than the optimal temperature for growth (Jobling, 1997).

Water temperature also influenced the dietary carbohydrate utilisation in rainbow trout (Brauge et al., 1995). Dietary imbalance in relation to temperature might be a factor of poor carbohydrate utilisation (Ringrose, 1971). It has been shown in gilthead sea bream and European sea bass that feeding excessive carbohydrate levels at low temperatures exhibited low carbohydrate assimilation due to low enzymatic activity, while coldwater species (Atlantic salmon, *Salmo salar*) can adapt their enzymatic digestion and utilise carbohydrate at low temperatures (Papoutsoglou and Lyndon, 2005). Furthermore, it was confirmed in Senegalese sole, *Solea senegalensis* that glycolysis was not affected by the starch level of diets and elevated levels of lipid inhibited lipid biosynthesis from carbohydrate (lipogenesis) (Dias et al., 2004) which was increased at lower temperature (Guerreiro et al., 2012a; Guerreiro et al., 2012b).

Histological features of the liver and gut of carnivorous fish fed different level of carbohydrate in relation to temperature is limited, however, data does exist for carp, *Catla catla* (Yengkokpam et al., 2005) and *Labeo rohita* (Mohapatra et al., 2003; Kumar et al., 2005). It was determined that fish fed either gelatinised or non-gelatinised carbohydrate did not affect the histological structure of the liver (Kumar et al., 2005). Furthermore, higher levels of gelatinised carbohydrate increased the hepatocyte hypertrophy and vacuolation (Mohapatra et al., 2003; Yengkokpam et al., 2005) and may be due to the storage of glycogen or lipid.

Like other salmonids, brook trout are susceptible to warmer temperatures, which reduce growth (Robinson et al., 2010). Brook trout inhabit water ranging from 0°C to 20°C (Power, 1980), although their preferable temperature has been shown to range from 11 to 19°C (Graham, 1949; Clements, 1988). Temperature dependant growth of brook

trout has been determined, where maximum growth of brook trout was observed at 13 - 14°C (Baldwin, 1957; McMahon et al., 2007; Fischer et al., 2009; Robinson et al., 2010). It performed poorly at temperatures over 20°C for extended periods and did not survive at 25°C for more than few hours (Raleigh, 1982). Optimum temperature for growth should not exceed 15.6°C (Raleigh, 1982). However, in Tasmania where brook trout are normally farmed, summer temperatures commonly reach to 19°C-20°C (Miller et al., 2006; Ng et al., 2010; Lough and Hobday, 2011). To the best of my knowledge, there are no studies available on the effect of temperatures on the nutrient utilisation in brook trout fed a carbohydrate based diet. Brook trout may show the potentiality to use gelatinised carbohydrate, therefore, the overall objectives of this study were to determine the effect of high carbohydrate in their feed on nutrient utilisation, health, protein and carbohydrate digestion, energy utilisation at ambient temperature (15°C) and high summer temperatures experienced in Australian salmonid production (19°C).

4.3. Materials and methods

4.3.1. Experimental diet

Four iso-nitrogenous (44% protein) and iso-energetic (22 MJ·kg⁻¹) diets with different lipid and carbohydrate level were prepared from dry ingredients (Table 4.1). The main protein sources (fish meal) and fish oil were supplied by Skretting (Cambridge, Tasmania, Australia). Carbohydrate was supplied as pre-gelatinised maize starch (BO11C). All the dietary ingredients were mixed thoroughly by a Brice mixer (Model: VFM – 20 C, Brice Australia Pty Ltd, Burwood, VIC) and approximately 12% water was added, then pelleted through 3 mm-die in a California Laboratory Pellet Mill (California Laboratory Pellet Mill Co., San Francisco, USA). The diets were dried in an oven (Model: 68732-1, Forma Scientific, Division of Mallinckrodt. INC. Marietta, Ohio, USA) to below 10% moisture content and stored at 2°C.

Table 4.1. The ingredient and chemical composition of experimental feeds (g·kg⁻¹)

Ingredient Inclusion	GCHO18	GCHO21	GCHO24	GCHO26
Fish meal	511	511	511	511
Wheat gluten	100	100	100	100
Fish oil	108.1	94.5	81	72
Pregelatinised maize starch	180	210	240	260
Vitamins ^a	15	15	15	15
Minerals ^b	15	15	15	15
Stay C	15	15	15	15
Choline chloride	1	1	1	1
Monobasic calcium phosphate	10	10	10	10
Ytterbium oxide	1	1	1	1
α -cellulose	44	27	11	0
<i>Chemical composition (Dry matter basis)</i>				
Dry matter	907.1	903.2	907.7	906.3
Crude protein	438.7	437.1	443.1	443.5
Crude lipid	167.9	155.3	140.2	131.1
Ash	109.4	106.7	110.1	107.4
NFE ^c	191.1	204.0	214.2	224.2
Gross energy (MJ·kg ⁻¹)	22.32	21.29	21.71	21.82
CHO:L	1.07	1.35	1.71	1.98

^a Vitamin premix (mg·kg⁻¹ of mixture) = Vitamin A acetate (ICN), 7.50; Vitamin D3 powder (ICN), 9.00; Rovimix E50, 150.00; Menadione sodium bisulfite, 3.00; Riboflavin, 6.00; Calcium D-pantothenate, 32.68; Nicotinic Acid, 15.00; Vitamin B12, 0.015; d-Biotin, 0.225; Folic Acid, 1.50; Thiamin HCL, 1.68; Pyridoxine HCL, 5.49; Myo-Inositol, 450.00; α -cellulose, 817.91; Stay-C, 150.00.

^b Mineral premix (mg·kg⁻¹ of mixture) = CuSO₄ 5H₂O (cupric sulphate), 35.37; FeSO₄ 7H₂O (ferrous sulphate), 544.65; MnSO₄ H₂O (manganese sulphate), 92.28; Na₂SeO₃ (sodium selenate), 0.99; ZnSO₄ 7H₂O (zinc sulphate), 197.91; KI (potassium iodide), 2.16; CoSO₄ 7H₂O (cobalt sulphate), 14.31; α -cellulose, 612.33.

^c NFE = 100 – (% moisture + % protein + % lipid + % ash)

4.3.2. Experimental system and design

The experiment had a 2 X 4 factorial design with two temperatures, four diets and was conducted in triplicate with a total of 24 tanks each with a 300L capacity. The trial was conducted in two independent freshwater recirculation systems at the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), University of Tasmania, Launceston, Australia. Each system was allocated a temperature either 15°C or 19°C. Each of the systems contained 12 (300L) tanks, arranged in two separate 4000 L systems, containing a biofilter and an UV light. Water temperature was controlled by a heat chiller unit and temperature was recorded hourly by temperature data logger (HOBO® Pendant, part# UA-002-XX, Onset Computer Corporation, Pocasset, USA). The average temperatures (mean±SD) were 15.06±0.39°C and 19.48±0.44°C, respectively. Water quality parameters (dissolved oxygen, pH, ammonia, nitrite and nitrate) were recorded daily and maintained within the limit for salmonids. Dissolved oxygen was recorded above 90% for this experiment.

4.3.3. Experimental fish and feeding trial

Fish were provided by Snowy Range Trout Fishery (Hobart, Tasmania, Australia) and were initially stocked in a 2000 L holding tank. Fish were randomly allocated to the 24 experimental tanks (31 fish·tank⁻¹) for acclimation. Fish were fed a 2 mm commercial rainbow trout diet (Spectra SS, Skretting, Cambridge, Tasmania, Australia) at 1% body weight per day for one week during acclimation. Temperature was maintained at 15°C in one system and slowly increased (1°C·d⁻¹) to 19°C in the other system. Before starting the experiment, individual fish were weighed (g) and lengths (cm) were measured. Twelve fish were randomly selected from each of the temperature system and sacrificed by overdose of anaesthesia (400 ppm, Aqui-S, AQUI-S New Zealand Ltd.) to determine the initial whole-body composition. Finally, thirty fish were returned to each tank. Fish were reared in the experimental systems for 12 weeks. During this period the fish were fed one of the four experimental diets at a uniform ration of 2% body weight per day which was equally divided in two feeds (9:30 and 15:30). Every 3 weeks fish were fasted for 24 h, anaesthetised with Aqui-S (100 ppm) and the bulk weight of fish in each tank was recorded. Rations were recalculated for the following 20

d period. Mortality was recorded during the experimental period and the ration was adjusted without replacing fish. At the end of the experiment, individual fish were weighed (g) and lengths (cm) were measured. Five fish from each of replicated unit (tank) were sacrificed by an overdose of the anaesthetic and frozen (-20°C) for later determination of body composition. Liver from three fish per tank were taken, halved and fixed with buffer formalin (pH 6.8) for final histology of liver. Pyloric caecae were dissected, fat removed and immediately frozen in liquid nitrogen and stored at -80°C for digestive enzyme analysis.

4.3.4. Apparent digestibility

The remaining fish in each treatment were fed the same experimental feeds for digestibility analysis for additional 18 d. Faeces was collected on day 12 & 18. Fish were anaesthetised and faeces were collected by stripping (Austreng, 1978) at the distal part of the small intestine 4 h after feeding. Faeces were frozen; freeze dried and stored at -20°C until chemical analysis. Apparent digestibility was calculated by using the formula:

Apparent digestibility of dry matter (AD_{DM})

$$= 100 - 100 \cdot (\% \text{ marker in feed} \cdot \% \text{ marker in faeces}^{-1}) \quad (\text{De Silva and Anderson, 1995})$$

Apparent digestibility (AD) of nutrient

$$= 100 - [100 \{ (\% \text{ marker in feed} \cdot \% \text{ marker in faeces}^{-1}) \cdot (\% \text{ nutrient in faeces} \cdot \% \text{ nutrient in feed}^{-1}) \}] \quad (\text{Maynard and Loosli, 1969})$$

4.3.5. Activity of α -amylase assay

Pyloric caecae were pooled per tank (n=3) for each dietary treatment for analysis. Frozen pyloric caecae were homogenised with 0.6M PCA with an electric homogeniser and centrifuged (3000 rpm for 20 min at 4°C). The supernatant was taken as an enzyme extract. Activity of α -amylase was determined by using Phadebas[®] test kit (Ceska et al., 1969). One Phadebas tablet was mixed with 10 ml 0.02 M sodium phosphate buffer (pH7) to prepare a dye-linked amylose suspension. Aliquots of enzyme extract (250µl) were incubated with 0.5ml amylose suspension for 60 min at 37°C and absorbance was measured at 620 nm. Blank sample was prepared without adding the enzyme extract.

The activity unit of α -amylase was expressed as an international unit (U), one unit of amylase was defined as the amount of enzyme catalysing the hydrolysis of one μmol glucosidic linkage per minute at 37°C .

4.3.6. Chemical analysis

Fish and feed samples were autoclaved (Williams et al., 1995) before freeze drying to constant weight, then homogenised for chemical analysis. Dry matter, ash, crude protein were analysed according to AOAC standard procedures (AOAC, 2005). Crude lipid was determined according to Bligh & Dyer (1959), crude protein by Kjeldahl method and gross energy through combustion in a calorimetric bomb (Gallenkamp Autobomb). To analyse the ytterbium, feed and faecal sample were homogenised then digested by using concentrated HNO_3 . The digestion procedure was done by adding 2 ml concentrated HNO_3 to the sample. Samples were digested at 90°C for 3 h. The digests were diluted (1:100, v/v) by distilled water and 10 ml of 10,000 ppm KNO_3 . Ytterbium content was analysed by Flame Atomic Absorption Spectrometry (Xplora AA, GBC Scientific Equipment, Australia) using nitrous oxide – acetylene flame (lamp current: 5 mA, wavelength: 398.8 nm and a slit width of 0.2 nm).

4.3.7. General histology

For histological studies, three livers and hind guts were taken from each of the replicated tank. Slices from the medium portion of liver were fixed in buffered formalin. Hind gut were exposed by needle and kept in fixative. Liver and gut samples were embedded in paraffin, cut at $5\ \mu\text{m}$, and stained with haematoxylin and eosin (H&E) (Roberts, 1989). The gut features were evaluated according to Baeverfjord & Krogdahl (1996) and Urán et al. (2008).

4.3.8. Calculations

The nutrient efficiency and performance indices were determined as follows:

Specific growth rate (SGR, $\% \text{ d}^{-1}$)

$$= 100 (\ln \text{ final weight} - \ln \text{ initial weight}) \cdot \text{day}^{-1} \quad (1)$$

$$\text{Feed efficiency ratio (FER)} = \text{weight gain} \cdot \text{dry feed fed}^{-1} \quad (2)$$

Productive Protein Value (PPV, %)

$$= (\text{protein gain in the body (wet)} \cdot \text{crude protein fed}^{-1}) \cdot 100 \quad (3)$$

Productive Lipid Value (PLV, %)

$$= (\text{lipid gain in the body (wet)} \cdot \text{lipid fed}^{-1}) \cdot 100 \quad (4)$$

Productive Energy Value (PEV, %)

$$= (\text{energy gain in the body (wet)} \cdot \text{energy fed}^{-1}) \cdot 100 \quad (5)$$

Hepatosomatic index (HSI, %)

$$= (\text{liver weight} \cdot \text{body weight}^{-1}) \cdot 100 \quad (6)$$

4.3.9. Statistical analysis

The data are presented as mean values \pm standard deviation (SD). Two-way analysis of variance (ANOVA) was performed by considering the diet and temperature as the two factors followed by Tukey's honestly significant difference (HSD) for multiple comparisons. Where there was no interaction between temperature and diet on the response on growth, feed utilisation, body composition; the diet factor was tested separately (one way ANOVA) at each temperature group followed by Tukey's test. Data were analysed using SPSS software package version 19.0 (SPSS Inc, IBM, IL, USA). The probabilities level of 0.05 or less were considered as significant for rejection of null hypothesis.

4.4 Results

4.4.1. Growth and feed utilisation indices

Initial weight (mean \pm SD) of fish was 48.03 ± 3.46 g and was not significantly different between temperatures or among dietary treatments. There was no significant interaction between temperature and diet on SGR ($F_{3,16}=0.640$, $P=0.600$), FER ($F_{3,16}=0.679$, $P=0.578$), PPV ($F_{3,16}=1.333$, $P=0.299$), PEV ($F_{3,16}=0.815$, $P=0.504$), and HSI, ($F_{3,16}=0.667$, $P=0.585$) (Table 4.2). The SGR, FER, PPV, PLV, PEV of brook trout were significantly higher at 15°C than 19°C (SGR, $F_{1,16}=66.92$, $P<0.001$; FER, $F_{1,16}=35.126$, $P<0.001$; PPV, $F_{1,16}=100.762$, $P<0.001$; PLV, $F_{1,16}=851.66$, $P<0.001$;

PEV, $F_{1,16}=174.115$, $P<0.001$). A significant interaction of rearing temperature and level of gelatinised carbohydrate was also found on PLV of brook trout ($F_{3,16}=45.563$, $P<0.001$), higher interaction was found in fish fed higher level of carbohydrate and reared at 15°C. The dietary effect on each parameter was tested by a one-way ANOVA separately at both temperatures followed by Tukey's test. No significant differences were found among the four dietary treatments either at 15°C or at 19°C in term of SGR ($F_{3,8}=0.943$, $P=0.464$ at 15°C; $F_{3,8}=1.336$, $P=0.329$ at 19°C) and FER ($F_{3,8}=1.274$, $P=0.347$ at 15°C; $F_{3,8}=0.977$, $P=0.450$ at 19°C) of brook trout. The PPV was significantly lower in fish fed lower carbohydrate diet (GCHO18) at both temperatures ($F_{3,8}=6.667$, $P=0.014$ at 15°C; $F_{3,8}=8.571$, $P=0.007$ at 19°C). The PEV was not affected by dietary treatment at 15°C ($F_{3,8}=3.774$, $P=0.059$), while there were significant differences at 19°C ($F_{3,8}=26.544$, $P<0.001$). The PEV was lower in the diet having low carbohydrate diet (GCHO18) in both temperatures. However, HSI of brook trout was not affected by dietary treatment at either temperatures ($F_{3,8}=0.667$, $P=0.596$ at 15°C; $F_{3,8}=0.667$, $P=0.596$ at 19°C).

4.4.2. Apparent digestibility

All of the apparent digestibility (%) parameters (Table 4.3) including apparent digestibility (AD) of drymatter (AD_{DM}), crude protein (AD_{CP}), crude lipid (AD_{CL}), gross energy (AD_{GE}) and energy from carbohydrate (energy obtained out of protein and lipid) (AD_{CHO-E}) were significantly higher at 15°C than 19°C (AD_{DM}, $F_{1,16}=451.227$, $P<0.001$; AD_{CP}, $F_{1,16}=235.767$, $P<0.001$; AD_{CL}, $F_{1,16}=81.000$, $P<0.001$; AD_{GE}, $F_{1,16}=657.565$, $P<0.001$; AD_{CHO-E}, $F_{1,16}=422.117$, $P<0.001$). There was a significant interaction between diet and temperature on the AD_{DM} ($F_{3,16}=3.331$, $P=0.046$), AD_{GE} ($F_{3,16}=9.318$, $P=0.001$) and on AD_{CHO-E} ($F_{3,16}=7.734$, $P=0.002$); and no interaction (diet x temp) was found on AD_{CP} ($F_{3,16}=0.603$, $P=0.622$) and AD_{CL} ($F_{3,16}=1.667$, $P=0.214$). By using a one-way ANOVA, the AD_{CP} and AD_{CL} were not affected by dietary treatment at both temperatures (AD_{CP} $F_{3,8}=2.011$, $P=0.191$ at 15°C, $F_{3,8}=1.085$, $P=0.409$ at 19°C; AD_{CL} $F_{3,8}=0.667$, $P=0.596$ at 15°C, $F_{3,8}=2.667$, $P=0.119$ at 19°C).

Table 4.2. Growth performance (mean \pm SD) and feed efficiency (mean \pm SD) of brook trout, *Salvelinus fontinalis* at two temperatures fed four experimental diets over 12 weeks

	15°C				19°C				Sig		
	GCHO18	GCHO21	GCHO24	GCHO26	GCHO18	GCHO21	GCHO24	GCHO26	Diet	Temp	Diet x Temp
Initial weight (g)	48.42 \pm 0.67 ^{aw}	47.89 \pm 2.31 ^{aw}	46.38 \pm 1.54 ^{aw}	49.13 \pm 0.97 ^{aw}	47.76 \pm 0.77 ^{aw}	48.85 \pm 2.10 ^{aw}	48.56 \pm 1.12 ^{aw}	47.25 \pm 0.80 ^{aw}	ns	ns	ns
Final weight (g)	177.40 \pm 39.41 ^{aw}	177.72 \pm 33.54 ^{aw}	180.37 \pm 34.04 ^{aw}	185.38 \pm 31.09 ^{aw}	152.82 \pm 41.97 ^{ay}	155.52 \pm 36.61 ^{ay}	158.18 \pm 31.19 ^{ay}	159.54 \pm 31.90 ^{ay}	ns	**	ns
SGR (% \cdot d ⁻¹)	1.60 \pm 0.04 ^{aw}	1.62 \pm 0.08 ^{aw}	1.68 \pm 0.07 ^{aw}	1.64 \pm 0.02 ^{aw}	1.44 \pm 0.02 ^{ay}	1.43 \pm 0.09 ^{ay}	1.46 \pm 0.02 ^{ay}	1.50 \pm 0.01 ^{ay}	ns	**	ns
FER	1.12 \pm 0.03 ^{aw}	1.15 \pm 0.06 ^{aw}	1.19 \pm 0.06 ^{aw}	1.16 \pm 0.02 ^{aw}	1.03 \pm 0.02 ^{ay}	1.01 \pm 0.09 ^{ay}	1.04 \pm 0.02 ^{ay}	1.08 \pm 0.00 ^{ay}	ns	**	ns
PPV (%)	42.46 \pm 1.17 ^{aw}	45.24 \pm 0.42 ^{bw}	46.06 \pm 0.94 ^{bw}	45.07 \pm 0.77 ^{bw}	38.89 \pm 0.65 ^{ay}	40.96 \pm 0.55 ^{by}	40.99 \pm 0.74 ^{by}	41.82 \pm 0.16 ^{by}	**	**	ns
PLV (%)	87.58 \pm 2.13 ^{aw}	93.2 \pm 0.18 ^{bw}	95.21 \pm 0.22 ^{cw}	97.30 \pm 0.68 ^{dw}	61.46 \pm 0.89 ^{ay}	72.52 \pm 2.21 ^{by}	83.85 \pm 1.32 ^{cy}	87.64 \pm 0.35 ^{dy}	**	**	**
PEV (%)	40.70 \pm 1.10 ^{aw}	44.29 \pm 2.15 ^{aw}	44.47 \pm 1.85 ^{aw}	43.34 \pm 0.61 ^{aw}	35.04 \pm 0.43 ^{ay}	38.12 \pm 0.5 ^{by}	37.11 \pm 0.64 ^{by}	37.85 \pm 0.21 ^{by}	**	**	ns
HSI (%)	2.30 \pm 0.22 ^{aw}	2.31 \pm 0.37 ^{aw}	2.62 \pm 0.32 ^{aw}	2.43 \pm 0.32 ^{aw}	2.18 \pm 0.30 ^{aw}	1.98 \pm 0.33 ^{aw}	2.35 \pm 0.29 ^{aw}	2.35 \pm 0.51 ^{aw}	ns	ns	ns

Specific growth rate (SGR, % \cdot d⁻¹) = 100 (ln final weight - ln initial weight) \cdot day⁻¹; Feed efficiency ratio (FER) = weight gain-dry feed fed⁻¹; Productive protein value (%PPV) = (protein gain in the body (wet) \cdot crude protein fed⁻¹) \cdot 100; Productive lipid value (%PLV) = (lipid gain in the body (wet) \cdot lipid fed⁻¹) \cdot 100; Productive energy value (%PEV) = (energy gain in the body (wet) \cdot energy fed⁻¹) \cdot 100; Hepatosomatic index (HSI, %) = (liver weight \cdot body weight⁻¹) \cdot 100.

Data were analysed by two-way ANOVA. Significant effects are presented at P<0.05 denoted by single asterisk, at P<0.001 denoted double asterisk and ns, not significant. One-way ANOVA was performed at both temperatures when an interaction between temperature and diet on the performances was not found followed by Tukey's test; different superscript letters a, b, c indicate that data were significantly different among the diets and w, y indicate that data were significantly different between temperatures; while same superscript letters indicate that there was no difference.

Table 4.3. Effect of temperature and varying dietary carbohydrate and lipid level on apparent digestibility for brook trout, *Salvelinus fontinalis*

Apparent digestibility (%)	15°C				19°C				Sig		
	GCHO18	GCHO21	GCHO24	GCHO26	GCHO18	GCHO21	GCHO24	GCHO26	Diet	Temp	Diet x Temp
AD _{DM}	77.61±0.18 ^{aw}	79.09±0.72 ^{bw}	81.66±1.03 ^{cw}	82.02±0.30 ^{cw}	68.98±0.80 ^{ay}	73.48±0.32 ^{by}	74.39±1.04 ^{cy}	74.47±0.44 ^{cy}	**	**	*
AD _{CP}	91.41±0.25 ^{aw}	91.56±0.09 ^{aw}	91.77±0.02 ^{aw}	91.45±0.29 ^{aw}	89.15±0.57 ^{ay}	89.65±0.49 ^{ay}	89.64±0.23 ^{ay}	89.65±0.10 ^{ay}	ns	**	ns
AD _{CL}	97.96±0.34 ^{aw}	98.29±0.38 ^{aw}	98.27±0.15 ^{aw}	98.35±0.12 ^{aw}	97.32±0.07 ^{ay}	96.75±0.17 ^{ay}	96.25±0.58 ^{ay}	96.36±0.24 ^{ay}	ns	**	ns
AD _{GE}	84.45±0.88 ^{aw}	85.22±0.05 ^{bw}	88.32±0.31 ^{cw}	87.96±0.43 ^{dw}	79.25±0.24 ^{ay}	80.10±0.45 ^{by}	80.35±0.56 ^{cy}	82.74±0.18 ^{dy}	**	**	**
AD _{CHO-E}	56.99±3.34 ^{aw}	57.49±0.51 ^{aw}	72.94±1.01 ^{bw}	73.06±1.39 ^{cw}	40.90±0.38 ^{ay}	41.75±1.78 ^{ay}	50.06±3.66 ^{by}	61.02±0.72 ^{cy}	**	**	*

Data were analysed by two-way ANOVA. Significant effects are presented at P<0.05 denoted by single asterisk, at P<0.001 denoted double asterisk and ns, not significant. One-way ANOVA was performed at both temperatures when interaction between temperature and diet on the performances was not found (P > 0.05) followed by Tukey's test; different superscript letters a, b, c indicate that data were significantly different among the diets and w, y indicate that data were significantly different between temperatures; while same superscript letters indicate that there was no difference.

4.4.3. Activity of α -amylase

The temperature had a significant ($F_{1,16}=145.392$, $P<0.001$) effect on the amylase activity of brook trout which was higher at 15°C. A significant interaction of rearing temperature and level of gelatinised carbohydrate was also found on amylase activity of brook trout ($F_{3,16}=5.705$, $P<0.001$), higher interaction was found in fish fed higher level of carbohydrate and reared at 15°C (Fig. 4.1).

4.4.4. Fish body composition

There was no interaction between temperature and diet on the moisture content of fish ($F_{3,16}=0.096$, $P=0.961$) (Table 4.4). However, the moisture content of fish was significantly higher at 19°C than 15°C ($F_{1,16}=27.716$, $P<0.001$), but was not affected by diet ($F_{3,16}=1.435$, $P=0.270$). The crude protein content of brook trout in this study was significantly affected by diet and temperature ($F_{3,16}=43.177$, $P<0.001$), this interaction was higher for diet GCHO26, having higher gelatinised carbohydrate (Table 4.4). A significant interaction was determined between temperature and diet for crude lipid ($F_{3,16}=4.339$, $P=0.020$) and ash ($F_{3,16}=53.741$, $P<0.001$) content (Table 4.4). Gross energy ($\text{MJ}\cdot\text{kg}^{-1}$) content of fish was affected by both diet and temperature ($F_{3,16}=4.260$, $P=0.022$), the interaction was lower for diet GCHO18, with lower gelatinised carbohydrate (Table 4.4). Overall, the gross energy content of fish was significantly higher at 15°C than 19°C ($F_{1,16}=2631.131$, $P<0.001$).

4.4.5. Histological feature of liver and gut

Liver histology was normal (Fig. 4.2) and there was moderate vacuolisation of hepatocytes of fish across all dietary treatments at both temperatures, due to glycogen storage confirmed by PAS staining (Fig. 4.3). Fish fed gelatinised carbohydrate based diets did not show enteritis-like changes to the intestinal epithelium at either temperature (Fig. 4.4). The signs of enteritis were scored according to Urán et al. (2008) featuring the reduction of supranuclear vacuolisation (SNV), abundance of goblet cells (GC), increased infiltration of eosinophilic granulocytes (EG), shrinkage of mucosal fold height (MF) increased width of lamina propria (LP) and cellular infiltration of the epithelial submucosa (SM).

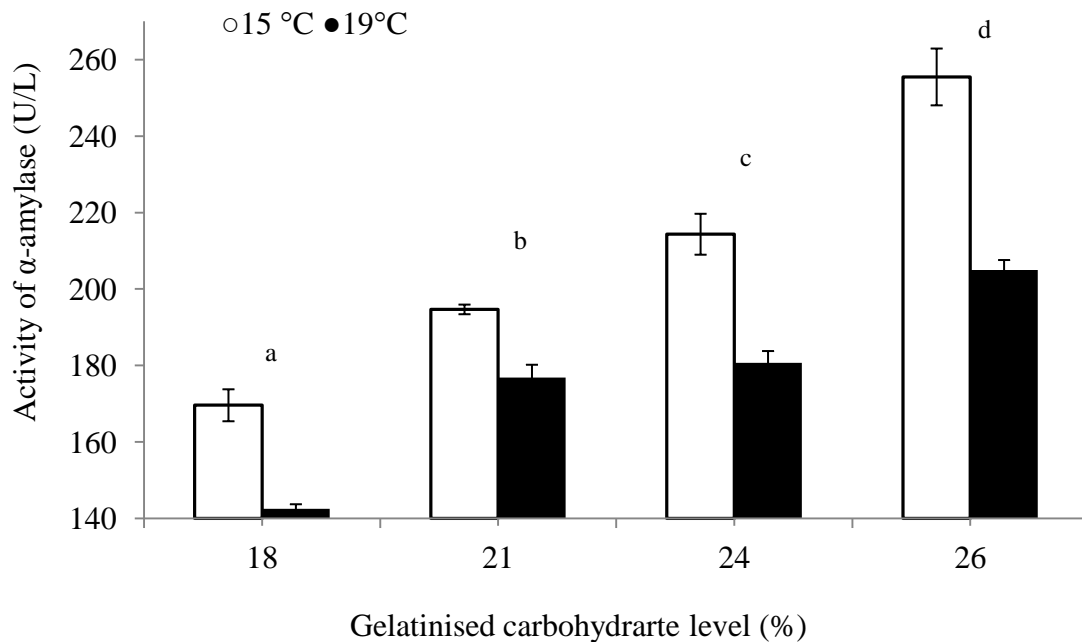


Figure 4.1. Activity of α -amylase activity of brook trout, *Salvelinus fontinalis* reared at 15°C or 19°C fed different level gelatinised carbohydrate based diet. One unit (U) of α -amylase was defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol glucosidic linkage per minute at 37°C. Data were analysed by two-way ANOVA, different superscript letters a, b, c and d indicate that diet x temperature interactions were significantly different.

Table 4.4. Proximate composition (g·kg⁻¹ wet weight) of brook trout *Salvelinus fontinalis* at two temperatures fed experimental diet over 12 weeks

	15°C				19°C				Sig		
	GCHO18	GCHO21	GCHO24	GCHO26	GCHO18	GCHO21	GCHO24	GCHO26	Diet	Temp	Diet x Temp
Moisture content	706.43±19.11 ^{aw}	703.63±13.73 ^{aw}	702.47±9.43 ^{aw}	703.08±10.02 ^{aw}	720.14±13.61 ^{ay}	713.77±7.77 ^{ay}	712.69±9.84 ^{ay}	714.95±8.91 ^{ay}	ns	**	ns
Crude protein	167.31±0.31 ^{aw}	169.29±0.68 ^{bw}	168.86±1.07 ^{bcw}	169.77±0.56 ^{cw}	167.79±0.51 ^{ay}	169.60±1.09 ^{by}	172.83±0.31 ^{bcy}	172.22±0.29 ^{cy}	**	**	**
Crude lipid	114.87±3.48 ^{aw}	115.50±4.37 ^{aw}	113.46±2.96 ^{aw}	107.42±1.19 ^{aw}	91.24±2.10 ^{ay}	96.54±5.46 ^{ay}	99.58±2.06 ^{ay}	96.26±2.24 ^{ay}	ns	**	*
Ash	22.60±0.25 ^{aw}	22.67±0.45 ^{aw}	25.01±0.48 ^{aw}	25.88±0.32 ^{aw}	23.89±0.26 ^{ay}	25.49±0.31 ^{ay}	21.31±1.15 ^{ay}	21.19±0.45 ^{ay}	ns	**	**
Gross energy (MJ·kg ⁻¹)	7.63±0.02 ^{aw}	7.69±0.04 ^{bw}	7.64±0.02 ^{bw}	7.67±0.03 ^{bw}	7.26±0.03 ^{ay}	7.30±0.01 ^{by}	7.32±0.02 ^{by}	7.32±0.01 ^{by}	**	**	*

Initial group (15°C): Moisture content, 737.26±11.56, Crude protein, 170.13±0.09, crude lipid, 70.96±3.15, ash content, 21.80±0.30, Gross energy (MJ·kg⁻¹), 6.35±0.06; (19°C): Moisture content, 733.09±6.76, Crude protein, 171.78±0.06, crude lipid, 70.96±2.15, ash content, 23.10±0.11, Gross energy (MJ·kg⁻¹), 6.50±0.00.

Data were analysed by two-way ANOVA. Significant effects are presented at P<0.05 denoted by single asterisk, at P<0.001 denoted double asterisk and ns, not significant. One-way ANOVA was performed at both temperatures when an interaction between temperature and diet on the performances was not found followed by Tukey's test; different superscript letters a, b, c indicate that data were significantly different among the diets and w, y indicate that data were significantly different between temperatures; while same superscript letters indicate that there was no difference.

Figure 4.2. Histological feature of liver brook trout, *Salvelinus fontinalis* fed carbohydrate based diet (scale = 50 μm). Fig. A-D: Fish fed diet GCHO18-GCHO26, respectively under the temperature 15°C and E-H fish fed diet GCHO18-GCHO26, respectively under the temperature 19°C.

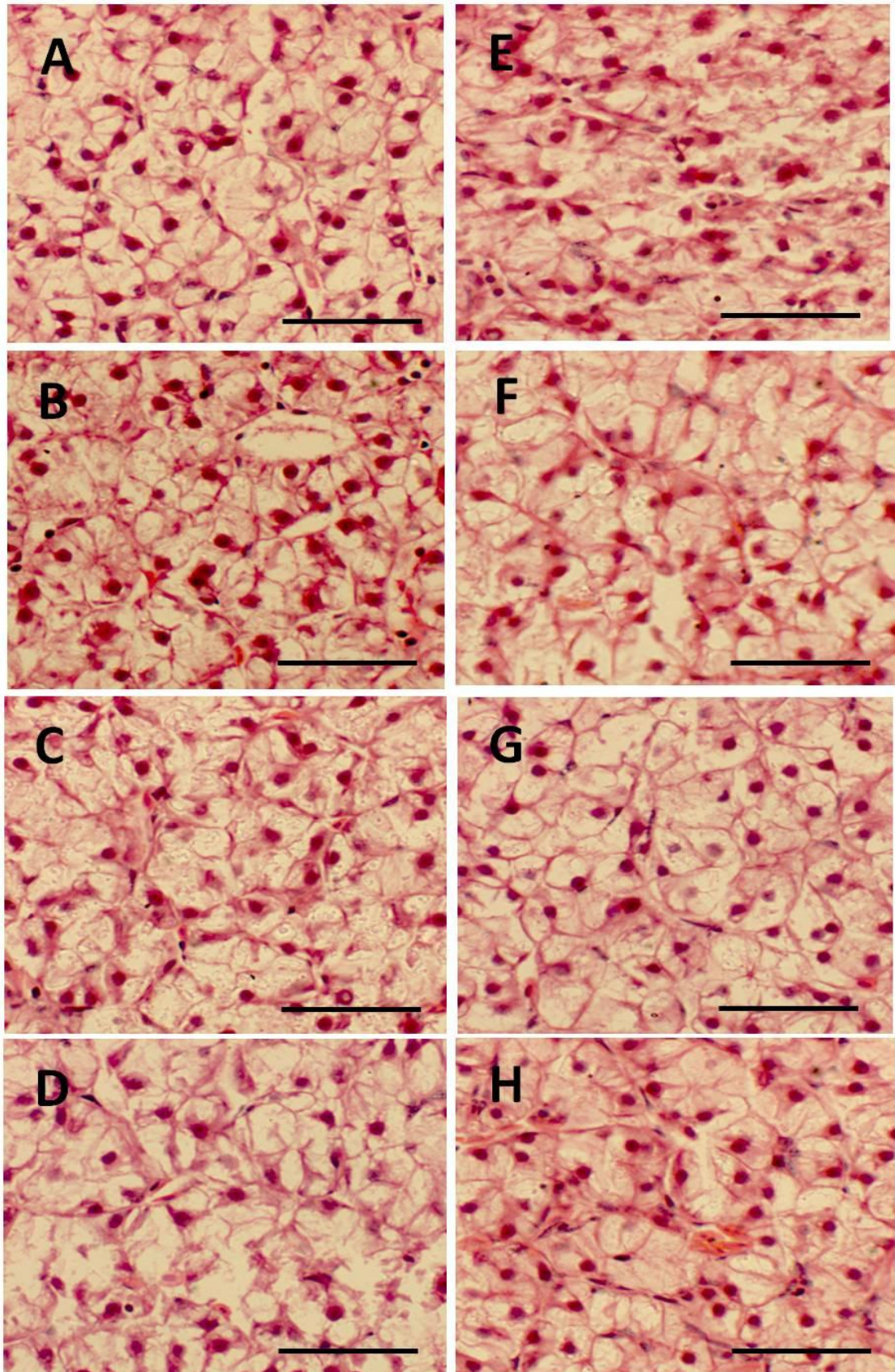


Figure 4.3. Light microscopic view of histochemical staining for glycogen within the hepatocyte of brook trout, *Salvelinus fontinalis*, fed different diets containing 26% gelatinised carbohydrate (scale = 50 µm). Section of liver stained with Periodic acid-schiff (PAS) and Haematoxylin (as counter stain), negative for PAS where slide was treated with salivary amylase. Glycogen in vacuole stained (PAS +ve) magenta colour (A), while not stained (PAS –ve) in control (B).

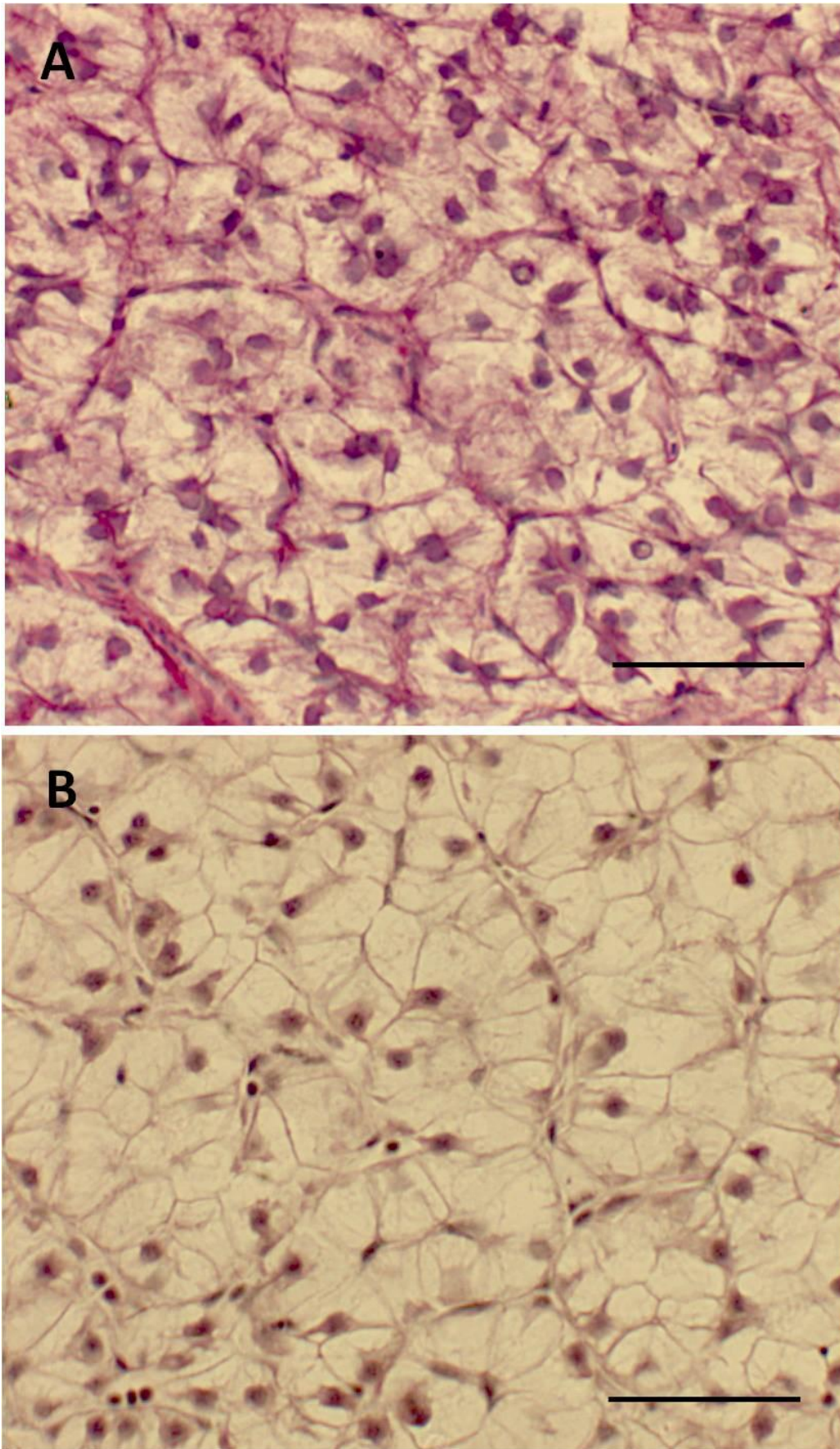
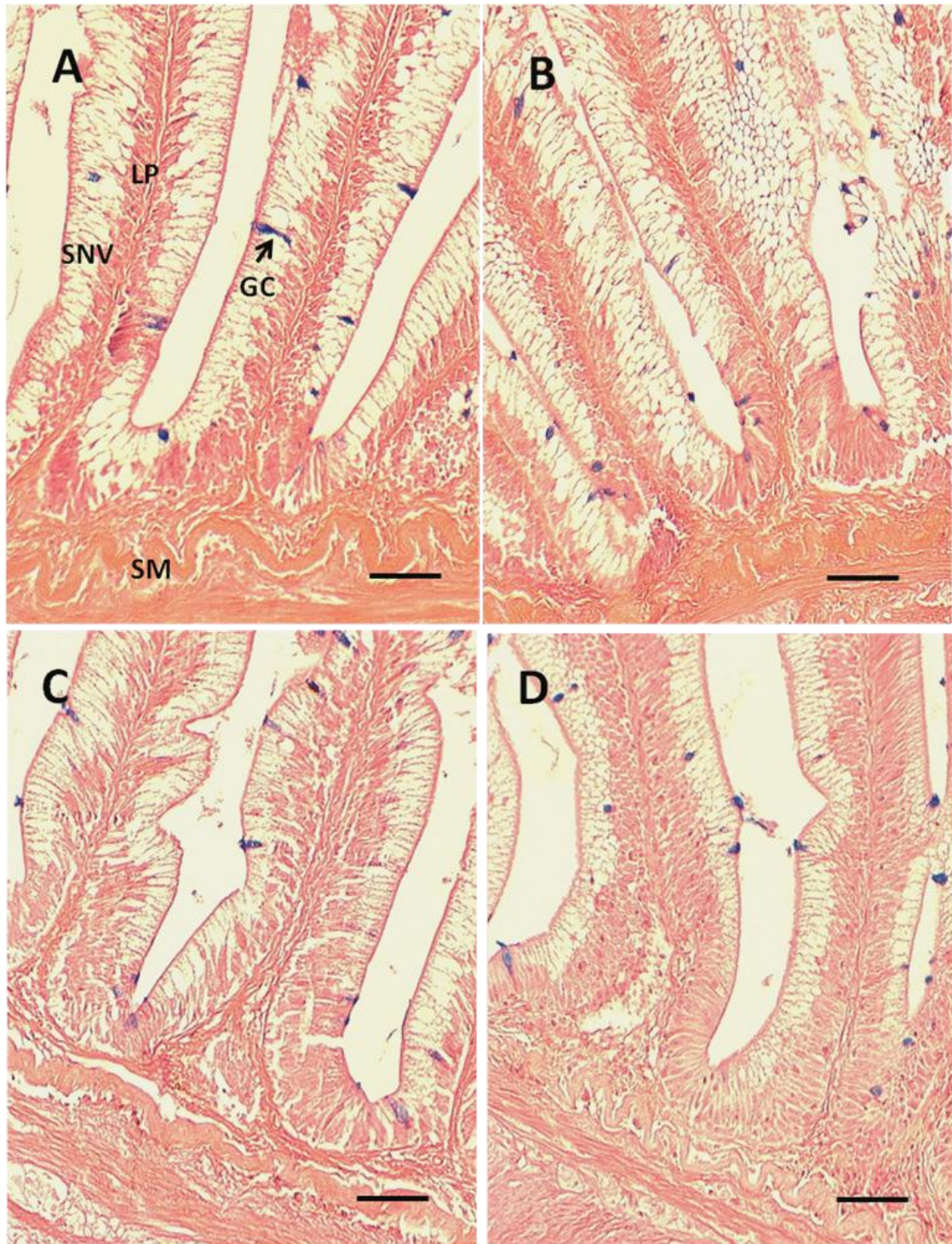


Figure 4.4. Histological feature of hind gut of brook trout, *Salvelinus fontinalis* fed carbohydrate based diet (scale = 50µm). Fig. A & B: Fish fed diet GCHO18 & GCHO26, respectively under the temperature 15°C and C & D fish fed diet GCHO18 & GCHO 26, respectively under the temperature 19°C. Supranuclear vacuoles SNV, goblet cells GC, lamina propria LP, sub-epithelial mucosa SM (H &E, Alcian blue staining). No distinct enteritis was found between the diet treatments (fed diet GCHO 21 & GCHO 24 are not shown in picture), SNV are normally aligned, scarcity of goblet cells, LP is thin, SM had normal size.



4.5. Discussion

To the best of my knowledge this is the first study to determine the effect of different levels of a gelatinised maize starch source of carbohydrate on the growth performances, feed utilisation, gut and liver histology of brook trout at two temperatures. Generally, neither high carbohydrate with low lipid nor low carbohydrate in the iso-energetic diet reduced the growth rate and feed utilisation at either temperature tested in this study, indicating that the same utilisation capacity of carbohydrate or lipid energy. Lower growth and feed utilisation in the brook trout diets at higher temperature is consistent with previous study (Chapter 2).

It was shown in the present study that increased carbohydrate can improve protein efficiency in brook trout fed the same level of protein with a lower lipid level, which can be explained as a protein sparing effect in these fish. Previous work has showed that increased levels of carbohydrate, with a lower protein level, successfully improved the PPV in brook trout, fed iso- energetic and iso- lipidic diet (Chapter 2). Higher inclusions of dietary carbohydrate lowered the protein utilised for energy and increased the protein efficiency for growth in Atlantic salmon and rainbow trout (Hemre et al., 1995; Grisdale-Helland and Helland, 1997; Krogdahl et al., 2004). In the present study, the lipid (PLV) and energy efficiencies (PEV) were increased with increasing carbohydrate. Therefore, brook trout can utilise energy from carbohydrate and lipid within the level of inclusion in the diet tested in this experiment. Further research needs to be done with higher carbohydrate and lower lipid beyond the level of this study to know the upper inclusion limit of carbohydrate in the diet of brook trout.

Temperature has a substantial effect on metabolism, feed intake, growth and the efficiency of nutrient utilisation (Jobling, 1997; Guerreiro et al., 2012b). In this study, nutrient utilisation such as protein, lipid and energy utilisation was higher at 15°C than 19°C indicating that an elevated summer temperature may reduce the nutrient utilisation in brook trout. The better nutrient utilisation at 15°C than 19°C may coincide with increased activity of digestive enzyme at lower temperatures. It was found in sea bream that gelatinised starch digestibility was not affected by temperatures due to the increased activity of amylase at low temperature to cope with temperature (Couto et al.,

2012). This was not the case in this study, indicating that brook trout are less capable of utilizing nutrient at elevated summer temperature. The effect of temperature on protein or nutrients utilisation in fish is contradictory (Olivia Teles and Rodrigues, 1993; Burel et al., 1996; Peres and Oliva-Teles, 1999; Guerreiro et al., 2012b). The efficiency of protein utilisation was higher at 18°C than 25°C in European sea bass (Peres and Oliva-Teles, 1999). In contrast, nutrient utilisation was more efficient at 21°C than 16°C in rainbow trout (Olivia Teles and Rodrigues, 1993) and the higher protein utilisation was found at 22°C than 16°C in Senegalese sole. However, protein efficiency was not affected by temperature in turbot (Burel et al., 1996). The variation of results in nutrient utilisation may be due to temperature and dietary ingredient digestibility of the studied fish. The nutrient utilisation was maximised at that temperature which was slightly lower than the optimum temperature for growth (Jobling, 1997) and best nutrient utilisation was also related to an improvement in diet digestibility (Olivia Teles and Rodrigues, 1993).

Hepatosomatic index (HSI) of brook trout fed iso energetic and iso-lipidic diets was increased with increased dietary carbohydrate level (Chapter 3; Moreira et al., 2008; Alexander et al., 2011). The higher HSI was due to an increase in liver size by reserving energy in the form of glycogen and/or as lipid in liver of fish fed high starch diet (Dias et al., 1998; Peres and Oliva-Teles, 2002; Moreira et al., 2008). An effect of carbohydrate was not observed in this study, possibly due to low lipid levels in the high carbohydrate diets and therefore excess carbohydrate was catabolised to compensate for energy.

Increased apparent digestibility of dry matter (AD_{DM}), gross energy (AD_{GE}) and carbohydrate energy (AD_{CHO-E}) with increased dietary carbohydrate levels at both temperature revealed that brook trout may compensate their energy requirement by utilizing more carbohydrate. The higher carbohydrate with low lipid in the diet of brook trout had no negative effect on the apparent digestibility of protein and lipid. High levels of carbohydrate may reduce the apparent digestibility of macronutrients though the reason is not fully explained (Olsen and Ringø, 1998). It may be due to crude starch absorbing amylase and inhibiting the starch hydrolysis and therefore reducing the digestibility (Spannhof and Plantikow, 1983). High levels of starch increase the volume

of intestinal juices and accelerate the passage of chyme through intestine almost twice as quickly as protein-rich diets and reduced the availability of time for absorption (Spannhof and Plantikow, 1983). Increased non-starch polysaccharide (NSPs) in the diet increases the digesta viscosity in the proximal and distal intestine, which reduces the protein and lipid digestibility (Sinha et al., 2011). Non-starch polysaccharides may entrap the bile salts; consequently reducing the solubility of fat and reduced lipid absorption (Sinha et al., 2011). Since salmonids have a relatively small intestinal surface area compared to cyprinids, poorer digestion obtained in salmonids may relate to the smaller degree of membrane contact for digestion, however, it did not seem to be involved in reduced digestibility of starch in rainbow trout (Spannhof and Plantikow, 1983). However, high levels of dietary starch significantly reduced the digestibility of protein, lipid, starch, energy and dry matter when compared to low levels of starch in Atlantic salmon; conversely, no differences were found in rainbow trout (Krogdahl et al., 2004). Trout have been shown to be more capable of utilising carbohydrate than salmon (Krogdahl et al., 2004). The higher digestibility of gelatinised carbohydrate is documented in rainbow trout (Inaba et al., 1963; Pieper and Pfeffer, 1980a; Bergot and Breque, 1983), while digestibility of gelatinised carbohydrate was decreased with increasing inclusion level in Atlantic salmon (Aksnes, 1995).

Water temperature has a significant effect on the apparent nutrient digestibility in salmonids (Watanabe et al., 1996; Azevedo et al., 1998; Olsen and Ringø, 1998; Bendiksen et al., 2003). Above 15°C protein digestibility was shown to decrease in rainbow trout (Watanabe et al. 1996; this study). Apparent digestibility of dry matter and protein were lower at 19°C than 15°C (Chapter 3). Likewise apparent digestibility of lipid, gross energy and carbohydrate energy was also lower at 19°C in this study. Protein digestibility was high (around 91%) in Atlantic salmon, reared at temperature ranged from 2°C to 8°C (Bendiksen et al., 2003). Arctic char, *Salvelinus alpinus* showed lower digestibility of dry matter and all nutrients at 0.6°C than at 10°C (Olsen & Ringø 1998). Apparent digestibility of dry matter and protein increased with temperature from AD_{DM} 73% and AD_{CP} 81% at 6°C to AD_{DM} 82% and AD_{CP} 89% at 15°C (Azevedo et al., 1998).

Plants contain varieties of amylase inhibitors (peptides or protein) (Franco et al., 2002), and both protein and hydrolysis products can inhibit the action of α -amylase on starch (Irshad and Sharma, 1981; Hill et al., 1997). This kind of inhibitor might not affect the digestibility in brook trout, since higher levels of gelatinised starch did not affect the digestibility. Amylase activity in brook trout fed gelatinised maize starch was positively related with starch level, which was found in many fish species (Krogdahl et al., 2005). In contrast, intestinal amylase activity was reduced in rainbow trout fed high level of raw corn starch (Spannhof and Plantikow, 1983). The lower activity of amylase also found in gilthead sea bream fed raw corn starch. Amylase inhibition by wheat starch reduced the starch digestibility in fish, particularly in carp, but less sensitive to rainbow trout (Spannhof and Plantikow, 1983; Hofer and Sturmbauer, 1985). However, by thermal gelatinisation, the carbohydrate's chains are more accessible than raw starch for the enzymes (Baks et al., 2007). It is well known in fish that gelatinised carbohydrate are more digestible than raw starch (Stone et al., 2003), which may correlate with the higher activity of amylase. Higher activity of amylase was found in fish fed higher level of gelatinised carbohydrate (Mohapatra et al., 2003; Yengkokpam et al., 2007; Alexander et al., 2011).

Whole-body dry matter, lipid and ash were not increased with higher dietary starch, while whole-body protein was increased with higher dietary carbohydrate level. Higher levels of protein in the fish were likely related to protein sparing effect of carbohydrate. Both the lipid and gross energy value were decreased at higher temperatures and may be explained by increase in the metabolic energy requirement (Jobling, 1997) and the inability for fish to consume enough energy to store lipid (Shearer, 1994; Katersky and Carter, 2007).

Intestine and liver function and structure of any aquaculture species are important to ensure effective nutrient digestion, absorption and utilisation of dietary ingredient. The liver had moderately vacuolated hepatocytes, however, no pathological signs were found in the liver of fish in this study. Brook trout fed different levels of pre-gelatinised maize starch did not show any of the signs of enteritis in distal intestine. Temperature did not affect the intestinal structure in this study. In salmonids, enteritis process might be more influenced at high temperature, while enteritis developed at a lower

temperature seemed to be delay (Urán et al., 2008), however, such type of effect was not found in this study. Brook trout may tolerate high levels of pre-gelatinised maize starch based diet without any detrimental effect on gut health.

Due to poorer digestion capability, and technical processing considerations crude starch content in commercial salmon feeds have a 20% upper inclusion limit (Pieper and Pfeffer, 1980b; Spannhof and Plantikow, 1983). The energy derived from carbohydrate in commercial feed could be increased by improving the level of gelatinisation achieved during extrusion or using with higher degrees of pre-gelatinisation. In this present study, iso-energetic ($22\text{MJ}\cdot\text{kg}^{-1}$), and iso-nitrogenous (44%) diet containing 18-26% of gelatinised maize starch showed no significant difference in growth. In addition, all the feed efficiencies and digestibility data revealed that brook trout fed the higher gelatinised based diets performed better without compromising the function of the liver and intestine, which more evident at 15°C than 19°C .

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CHAPTER 5

PROTEIN AND ENERGY REQUIREMENTS OF BROOK TROUT, *SALVELINUS*
FONTINALIS (MITCHILL, 1814) AT TWO TEMPERATURES USING FACTORIAL
MODEL

5.1. Abstract

Elevated temperature is expected to increase protein and energy requirements of fish. Factorial modelling was used to determine the protein and energy requirements of brook trout (*Salvelinus fontinalis*) reared at either 15°C or 19°C. The digestible protein (DP) and digestible energy (DE) requirements for maintenance and growth were measured by feeding triplicate groups of fish at four rations (0%, 1%, and 2% of initial body weight and to satiety) at two temperatures (15°C or 19°C). The maintenance requirements for DP and DE were $0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ (15°C) and $0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ (19°C) and $29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ (15°C) and $36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ (19°C). The total requirements of DP ($\text{gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$) for growth were estimated to be $0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} + 2.14 \times \text{protein gain}$ (15°C) and $0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} + 1.99 \times \text{protein gain}$ (19°C). The total requirements of DE ($\text{kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$) for growth were estimated to be $29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} + 1.58 \times \text{energy gain}$ (15°C) and $36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} + 1.65 \times \text{energy gain}$ (19°C). The partial efficiency for growth in brook trout was 0.47 (15°C) and 0.50 (19°C) for protein and 0.63 (15°C) and 0.61 (19°C) for energy. Nutrient gain (protein or energy) was lower at elevated temperature; however, the relative response of nutrient gain was similar at both temperatures as the nutrient intake increase indicated that brook trout are equally capable of using dietary nutrients at both temperatures to achieve the same nutrient efficiency. Feed formulation for brook trout needs to be adjusted with their nutrient requirements to maximise growth with increasing culture temperatures and these models can be used to develop feeding charts for brook trout in commercial farms.

5.2. Introduction

Climate change is expected to affect the aquaculture industry, including salmon and trout farming (Battaglione et al., 2008; Lorentzen, 2008; Hobday et al., 2011). Salmonids are cold water species and are consequently more susceptible to the predicted effects of climate change (Robinson et al., 2010; Barnes et al., 2011). In many countries where salmon are farmed, water temperatures are increasing (Lorentzen, 2008; Ng et al., 2010; Lough and Hobday, 2011), however, southern hemisphere salmonid farms experience temperatures that are towards their upper thermal tolerance limit (Battaglione et al., 2008; Pankhurst and King, 2010; Barnes et al., 2011). It is widely accepted that increased temperature increases the energy demand as well as metabolism and reduces the growth of fish (Jobling, 1997; Miller et al., 2006; Katersky and Carter, 2007). Fish growth performance is being pushed harder at high temperature and aquafeeds need to be formulated to more closely meet the actual nutritional requirements (Carter et al., 2005). Although the nutrition of salmonids has been extensively studied, there is little information about the nutritional requirements of brook trout, *Salvelinus fontinalis* (Jobling et al., 2010). Like other salmonids brook trout are cold water species with an optimum temperature for growth of 14-16°C (Clements, 1988; McMahon et al., 2007). Within Australia, where brook trout are farmed, summer water temperatures are increasingly becoming elevated and often reach 19°C (Miller et al., 2006; Ng et al., 2010; Hobday and Lough, 2011; Lough and Hobday, 2011).

As fish grow, their energy and protein requirements change and therefore, should be estimated across their life cycle and in relation to the required level of performances and feed composition (Bureau and Hua, 2008). Since large fish contain more energy than small fish in term of per unit of biomass, the recovered energy can serve to determine the energy retention efficiency across the life cycle (Dumas et al., 2010). Factorial models have been used to determine protein and energy requirements across the size range required for commercial production of several fish (Glencross, 2008; Lupatsch, 2009; Booth et al., 2010; Amrkolaie et al., 2012). The advantages of using factorial model are that the nutrient requirements are calculated based on the daily feed requirement per unit of weight gain rather than expressed as percentage of the diet (Hauler and Carter, 2001; Lupatsch, 2009; Amrkolaie et al., 2012). The daily

requirement of protein and energy and protein for fish can be calculated by the following equation (Lupatsch et al., 2001a; Glencross and Bermudes, 2010; Pirozzi et al., 2010a):

$$\text{Total daily requirement} = a \times \text{BW (kg)}^b + c \times \text{gain}$$

Where “a” is the utilisation efficiency for maintenance as expressed in per unit of metabolic weight for certain conditions such as temperature for certain fish species, “b” is the exponent for metabolic body weight and “c” is the utilisation efficiency for protein or energy to obtain growth. The exponent “b” is average 0.80 and 0.70 for energy and protein requirement, respectively, can be used as common value for fish (Lupatsch, 2009). This above formula can be divided in to two as follows:

$$\text{Maintenance requirement} = a \times \text{BW (kg)}^b$$

and

$$\text{Requirement for growth} = c \times \text{gain}$$

Nutrient requirements of fish depend on various factors including species and temperature (Tacon and Cowey, 1985; Wilson and Halver, 1986; Jobling, 1994). Nutrient requirements of fish have usually been determined at optimum temperature where fish exhibit the best growth, however, have also been determined at above the optimum temperature for Atlantic salmon (Carter et al., 2005; Carter et al., 2008). According to available literature the optimum temperature for brook trout was approximately 15°C and a number studies have investigated the effect of temperature on the growth of brook trout under at or below this temperature (Baldwin, 1957; Gunther et al., 2007; Fischer et al., 2009). Protein and energy requirements are generally higher at high temperature and it is important to understand nutrition at more extreme temperature (Carter et al., 2005; Lupatsch, 2009). Compared even to other salmonids, brook trout are hardy species and adapts easily to culture condition (Jobling et al., 2010). Thus, the nutritive response of brook trout to high temperature may not be similar to salmon and there is real need to understand the growth and nutrition of brook trout at high temperature. By using factorial model, this study mainly aimed to

determine which feed composition and feeding rate are more suitable to use at 15°C and 19°C for brook trout during normal growth cycle in farm.

5.3. Materials and methods

5.3.1. General method

Experiments were conducted in freshwater recirculation systems with controlled water temperature (15°C and 19°C), at the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), University of Tasmania, Launceston. Water temperature was controlled by a heat chiller unit and temperature was recorded hourly by a data logger (HOBO® Pendant, part# UA-002-XX, Onset Computer Corporation, Pocasset, USA). Water quality parameters (dissolved oxygen, pH, ammonia, nitrite and nitrate) were recorded daily and maintained within the limit for salmonids. Dissolved oxygen was recorded above 90% for this experiment. Prior to sampling, fish were anaesthetised using isoeugenol (Aqui-S, 100ppm, AQUI-S New Zealand Ltd). Before starting each trial, fish were acclimated to the experimental systems for one week and water temperature was maintained at 15°C for both systems. Temperature was then maintained at 15°C in one system and slowly increased (1°C.d⁻¹) to 19°C in the other system. Fish in all trials (except the digestibility trial) were fed with commercial rainbow trout diet (Spectra SS, Skretting, Cambridge, Tasmania, Australia) at 1% of body weight (Rasmussen and Ostensfeld, 2010) a day during the time of acclimation.

5.3.2. Feeds

An experimental diet was formulated to be approximately 440 g·kg⁻¹ protein, 21 MJ·kg⁻¹ energy from major ingredient of fish meal, wheat gluten, fish oil and pre-gelatinised maize starch (B011C) (Table 5.1). Ytterbium oxide was added as an inert marker to the diet (10 g·kg⁻¹) at the start of feed preparation. All dry ingredients were mixed thoroughly by a Brice mixer (Model: VFM – 20C, Brice Australia Pty Ltd.) and approximately 12% water was added. The 2 mm pellets were made using a California Laboratory Pellet Mill (California Laboratory Pellet Mill Co., San Francisco, USA) and dried (Oven, Model: 68732-1, Forma Scientific, Division of Mallinckrodt. INC. Marietta, Ohio, USA) to below 10% moisture content and stored at 2°C until use.

Table 5.1. The ingredient and chemical composition of experimental feed ($\text{g}\cdot\text{kg}^{-1}$)

Ingredient	Inclusion
Fish meal	511
Wheat gluten	100
Fish oil	72
Pre-gelatinised maize starch	260
Vitamins ^a	15
Minerals ^b	15
Stay C	15
Choline chloride	1
Monobasic calcium phosphate	10
Ytterbium oxide	1
<i>Chemical composition (Dry matter)</i>	
Dry matter	919.1
Crude protein	438.9
Total lipid	158.8
Ash	99.5
Gross energy ($\text{MJ}\cdot\text{kg}^{-1}$)	20.89

^a Vitamin premix ($\text{mg}\cdot\text{kg}^{-1}$ of mixture) to supply per kg feed (Carter et al., 2003) = Vitamin A acetate (ICN), 7.50; Vitamin D3 powder (ICN), 9.00; Rovimix E50, 150.00; Menadione sodium bisulfite, 3.00; Riboflavin, 6.00; Calcium D-pantothenate, 32.68; Nicotinic Acid, 15.00; Vitamin B12, 0.015; d-Biotin, 0.225; Folic Acid, 1.50; Thiamin HCL, 1.68; Pyridoxine HCL, 5.49; Myo-Inositol, 450.00; α -cellulose, 817.91; Stay-C, 150.00.

^b Mineral premix ($\text{mg}\cdot\text{kg}^{-1}$ of mixture) to supply per kg feed (Carter et al., 2003) = $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (cupric sulphate), 35.37; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (ferrous sulphate), 544.65; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (manganese sulphate), 92.28; Na_2SeO_3 (sodium selenate), 0.99; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (zinc sulphate), 197.91; KI (potassium iodide), 2.16; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (cobalt sulphate), 14.31; α -cellulose, 612.33.

5.3.3. Starvation

The experiment was conducted in 24 tanks of 300L capacity of each, in two independent freshwater recirculation systems. Each system contained 12 tanks, arranged in two separate 4000 L systems with mean (\pm SD) temperatures of $14.85 \pm 0.33^{\circ}\text{C}$ and $19.09 \pm 0.52^{\circ}\text{C}$, respectively. The range of pH was 6.6 to 7.2, ammonia was 0 to 0.25 ppm, nitrite was 0 ppm and nitrate was 0-20 ppm.

Comparative carcass analysis was used to determine loss of weight, protein and energy. Fish of different weight classes (5 g, 10 g, 20 g, 200 g and 500 g) were stocked in duplicate tanks in the two separate systems for acclimation to experimental conditions. After acclimation fish were anaesthetised using isoeugenol (Aqui-S, 100ppm, AQUI-S New Zealand Ltd) and individually weighed (g) and length measured (cm). Ten fish were randomly selected from each tank and sacrificed by overdose of anaesthesia (Aqui-S, 400ppm, AQUI-S New Zealand Ltd) to determine the initial whole-body chemical composition. Fifteen fish from each size class were left unfed in duplicated tanks for 28 d. At the end of the experiment, fish were anaesthetised and individually reweighed and length measured. Ten fish from each of tank were sacrificed for whole-body chemical composition. Fish were frozen at -20°C until analysis. Frozen fish from each tank were pooled, minced and then analysed.

5.3.4. Apparent digestibility

Before stocking, fish were fed to satiation twice a day for two weeks with the experimental diet and then acclimated to the experimental system for one week. Following the acclimation period, twelve fish ($196.49 \pm 19.55\text{g}$) were stocked in triplicate tanks at each temperature. The average temperatures were $14.75 \pm 0.21^{\circ}\text{C}$ and $19.14 \pm 0.52^{\circ}\text{C}$, respectively. Fish were fed to satiation twice daily for ten days before faecal samples were taken. Fish were anaesthetised and faeces were collected by stripping the distal section of the small intestine 18 h after feeding (Percival et al., 2001; Ward et al., 2005). Faeces were frozen, freeze dried and stored at -20°C for chemical analysis. Apparent digestibility coefficients of protein and energy were calculated using the formula:

Apparent digestibility coefficient =

$$100 - [100 \{(\% \text{ marker in feed} \cdot \% \text{ marker in faeces}^{-1}) \times (\% \text{ nutrient in faeces} \cdot \% \text{ nutrient in feed}^{-1})\}]$$

(Maynard and Loosli, 1969)

5.3.5. Growth experiment

Fifteen fish were randomly allocated to each experimental tank. Fish were acclimated to experimental tank and feed for one week. After acclimation, twelve fish were randomly removed from each temperature system and sacrificed for initial whole-body chemical composition. Fourteen fish (21.70 ± 3.79 g) were re-stocked in each of twelve tanks at 15°C or 19°C. The average temperatures were $14.72 \pm 0.28^\circ\text{C}$ and $19.33 \pm 0.54^\circ\text{C}$, respectively. The ration was randomly assigned in triplicate and fish were either unfed, or fed twice daily at 1%, 2% of initial body weight, to satiety. Uneaten pellets were collected, counted and used to calculate the ration consumed. After 14 d, bulk weight of fish was taken to adjust the ration. At the end of the 28d trial, fish were individually weighed (g) and length measured (cm) and ten fish from each of replicated tank were sacrificed for whole-body chemical composition. Fish were frozen at -20°C until analysis. Frozen fish from each replicated tank were pooled, minced and then analysed for dry matter, protein, lipid and energy content.

5.3.6. Growth model

A potential growth model for brook trout was established by using weight increment data from a commercial farm. The growth data of brook trout from three consecutive years were used to calculate daily growth rate ($\text{g} \cdot \text{d}^{-1}$). The daily growth rate of brook trout was described using allometric equation, which was:

$$y = ax^b \tag{1}$$

Where, y = daily growth rate ($\text{g} \cdot \text{d}^{-1}$), x = body weight (BW) of fish (g), a = constant/slope and b = exponential.

5.3.7. Chemical analysis

Fish were homogenised, freeze dried to constant weight and then used for chemical analysis. Dry matter, ash, crude protein were analysed according to AOAC standard procedures (AOAC, 2005). Total lipid was determined according to Bligh and Dyer (1959), crude protein by Kjeldahl method and gross energy through combustion in a calorimetric bomb (Gallenkamp Autobomb). To analyse ytterbium, feed and faecal samples were homogenised and digested in 2 ml concentrated HNO₃ at 90°C for 3 h. The digests were diluted (1:100, v/v) with distilled water and 10 ml of 10,000 ppm KNO₃. Ytterbium content was analysed by Flame Atomic Absorption Spectrometry (XploraAA, GBC Scientific Equipment, Australia) using nitrous oxide – acetylene flame (lamp current: 5 mA, wavelength: 398.8 nm and a slit width of 0.2 nm).

5.3.8. Calculations:

The protein (g·fish⁻¹·d⁻¹) and energy (kJ·fish⁻¹·d⁻¹) intake were calculated digestible basis intake basis. The daily protein and energy gain were calculated by the following equations:

$$\begin{aligned} \text{Protein gain (g·fish}^{-1}\cdot\text{d}^{-1}) &= \\ (\text{final carcass protein} - \text{initial carcass protein}) \cdot \text{days}^{-1} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Energy gain (kJ·fish}^{-1}\cdot\text{d}^{-1}) &= \\ (\text{final carcass energy} - \text{initial carcass energy}) \cdot \text{days}^{-1} \end{aligned} \quad (3)$$

All the values obtained from those equations were then expressed per metabolic body weight of kg^b, where the exponent “b” was obtained from the starvation experiment. Metabolic body weight was calculated from the geometric mean of initial and final weight (initial body weight x final body weight)^{0.5}.

5.3.9. Statistical analysis

All data means \pm standard error (SE) were presented, unless specified otherwise. Linear regression better estimated the requirement compared to ANOVA (Hauler and Carter, 2001) and was applied to analyse the response of protein and energy retention against their intake (Bureau et al., 2002; Lupatsch, 2009). If the regressions were not linear, allometric equations were prepared (Bureau et al., 2002; Lupatsch, 2009) using SPSS software package version 20.0 (SPSS Inc, IBM, IL, USA) and used for analysing the data. All graphical presentations were performed using Microsoft Excel. The probability level of 0.05 or less was considered significant for rejection of the null hypothesis. Fish weights were converted to metabolic weight of the geometric mean of initial and final weight (initial body weight \times final body weight)^{0.5} to calculate nutrient retention. Raw data were presented at the appendices section (A-F).

5.4. Results

5.4.1. Metabolic weight exponent

Protein and energy loss was higher in larger fish than small fish and there was a declining trend with increasing weight (Fig. 5.1 & 5.2). Thus, the energy and protein loss was not a linear function of body weight and was described by allometric equations:

$$\begin{aligned} \text{Energy loss at } 15^{\circ}\text{C (kJ}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}) &= \\ 21.95 \pm 3.23 \text{ BWkg}^{0.84 \pm 0.04}, (R^2=0.98, F_{1,8}=448.52, P<0.001) \end{aligned} \quad (4)$$

$$\begin{aligned} \text{Energy loss at } 19^{\circ}\text{C (kJ}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}) &= \\ 27.11 \pm 2.53 \text{ BWkg}^{0.86 \pm 0.03}, (R^2=0.99, F_{1,8}=1203.09, P<0.001) \end{aligned} \quad (5)$$

$$\begin{aligned} \text{Protein loss at } 15^{\circ}\text{C (g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}) &= \\ 0.16 \pm 0.03 \text{ BWkg}^{0.69 \pm 0.04}, (R^2=0.97, F_{1,8}=238.18, P<0.001) \end{aligned} \quad (6)$$

$$\begin{aligned} \text{Protein loss at } 19^{\circ}\text{C (g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}) &= \\ 0.28 \pm 0.04 \text{ BWkg}^{0.78 \pm 0.04}, (R^2=0.98, F_{1,8}=457.04, P<0.001) \end{aligned} \quad (7)$$

The exponent of protein and energy loss (BW)^b can describe the metabolic body weight for protein and energy of brook trout. The observed exponent values were not

significantly different ($T=0.693$, $df=1$, $P=0.614$ for the protein exponent, $T=5.111$, $df=1$, $P=0.123$ for the energy exponent) from expected value, 0.70 and 0.80, respectively, generally used for fish (Lupatsch, 2009). Thus, 0.70 and 0.80 were used as metabolic weight exponent for protein and energy, respectively to calculate maintenance protein and energy requirement of brook trout in metabolic weight basis.

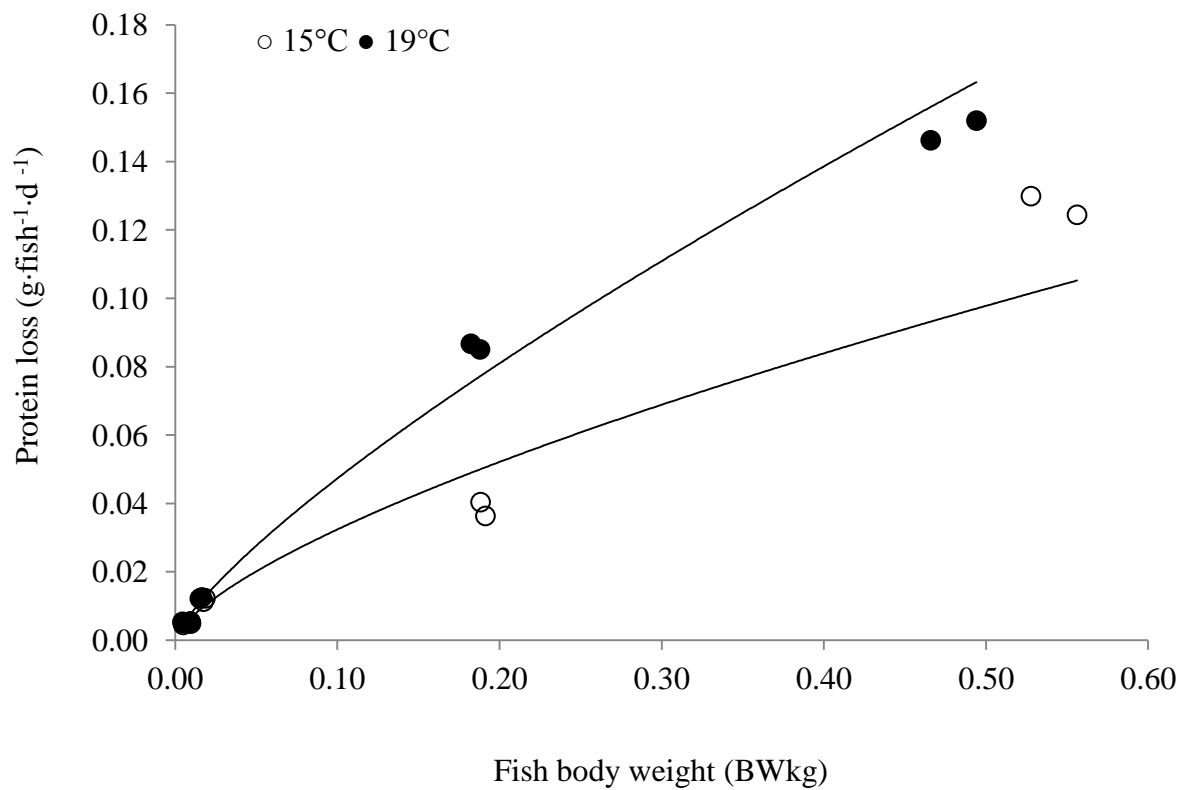


Figure 5.1. Protein loss ($\text{g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) in brook trout starved for 28 days at 15°C or 19°C. All equations are described by protein loss 15°C ($\text{g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) = $0.16 \pm 0.03 \text{ BWkg}^{0.67 \pm 0.04}$, ($R^2=0.97$, $F_{1,8}=238.18$, $P<0.001$) and protein loss 19°C ($\text{g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) = $0.28 \pm 0.04 \text{ BWkg}^{0.78 \pm 0.04}$, ($R^2=0.98$, $F_{1,8}=457.04$, $P<0.001$). Fish weights were converted to geometric mean of initial and final weight.

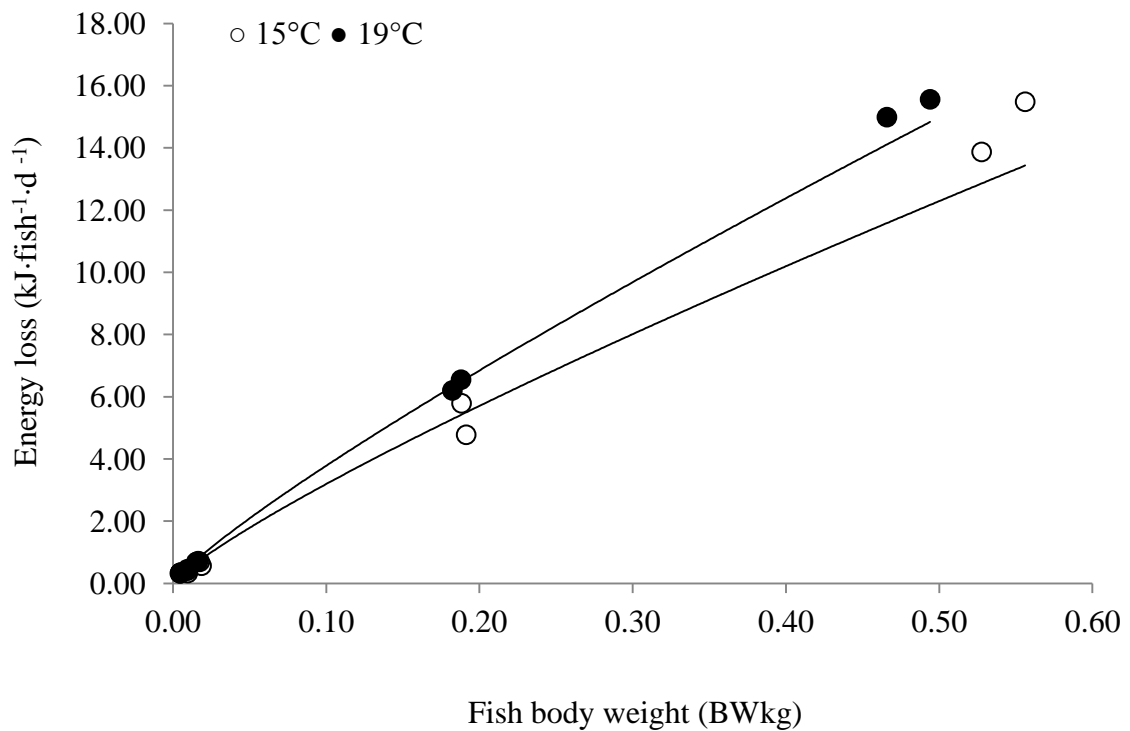


Figure 5.2. Energy loss ($\text{kJ}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) in brook trout starved for 28 days at 15°C or 19°C . All equations are described by energy loss 15°C ($\text{kJ}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) = $21.95 \pm 3.23 \text{ BWkg}^{0.84 \pm 0.04}$, ($R^2=0.98$, $F_{1,8}=448.52$, $P<0.001$) and energy loss 19°C ($\text{kJ}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}$) = $27.11 \pm 2.53 \text{ BWkg}^{0.86 \pm 0.03}$, ($R^2=0.99$, $F_{1,8}=1203.09$, $P<0.001$). Fish weights were converted to geometric mean of initial and final weight.

5.4.2. Protein and energy digestibility

The protein digestibility value of the experimental diet at 15°C and 19°C was $91.46 \pm 0.16\%$ and $88.95 \pm 0.23\%$, respectively and significantly higher at 15°C ($R^2=0.95$, $F_{1,4}=81.59$, $P<0.001$). The energy digestibility value of the experimental diet was $87.43 \pm 0.73\%$ and $83.66 \pm 0.56\%$ at 15°C and 19°C, respectively and significantly higher at 15°C ($R^2=0.81$, $F_{1,4}=16.784$, $P<0.001$).

5.4.3. Nutrient efficiency and requirement

The protein gain was modelled with linear regression against digestible protein intake (DPI) can be described by the following equation (Fig. 5.3):

$$\begin{aligned} \text{Protein gain at 15°C (g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}) = \\ 0.47 \pm 0.02\text{DPI (g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}) - 0.05 \pm 0.05 \quad (R^2=0.98, F_{1,10}=390.49, P<0.001) \end{aligned} \quad (8)$$

$$\begin{aligned} \text{Protein gain at 19°C (g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}) = \\ 0.50 \pm 0.04\text{DPI (g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}) - 0.11 \pm 0.06 \quad (R^2=0.95, F_{1,10}=211.72, P<0.001) \end{aligned} \quad (9)$$

Maintenance digestible protein requirement (DP_{maint}) of brook trout at both temperatures was calculated as the amount of digestible protein required (x) at zero retention of protein ($y = 0$),

$$\text{DP}_{\text{maint}} \text{ at 15°C} = 0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} \quad (10)$$

$$\text{DP}_{\text{maint}} \text{ at 19°C} = 0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} \quad (11)$$

The slope of linear regression calculates the partial retention efficiency of protein (K_{DP}), which was 0.47 and 0.50 at 15°C and 19°C, respectively. The reciprocal ($1/K_{\text{DP}}$) describes cost of digestible protein per unit of protein deposited. The $1/K_{\text{DP}}$ was 2.14 and 1.99 at 15°C and 19°C, respectively.

Thus, the daily protein requirement (DP_{total}) for brook trout is:

$$\text{DP}_{\text{total}} \text{ at 15°C} = 0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} + 2.14 \times \text{protein gain} \quad (12)$$

$$\text{DP}_{\text{total}} \text{ at 19°C} = 0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1} + 1.99 \times \text{protein gain} \quad (13)$$

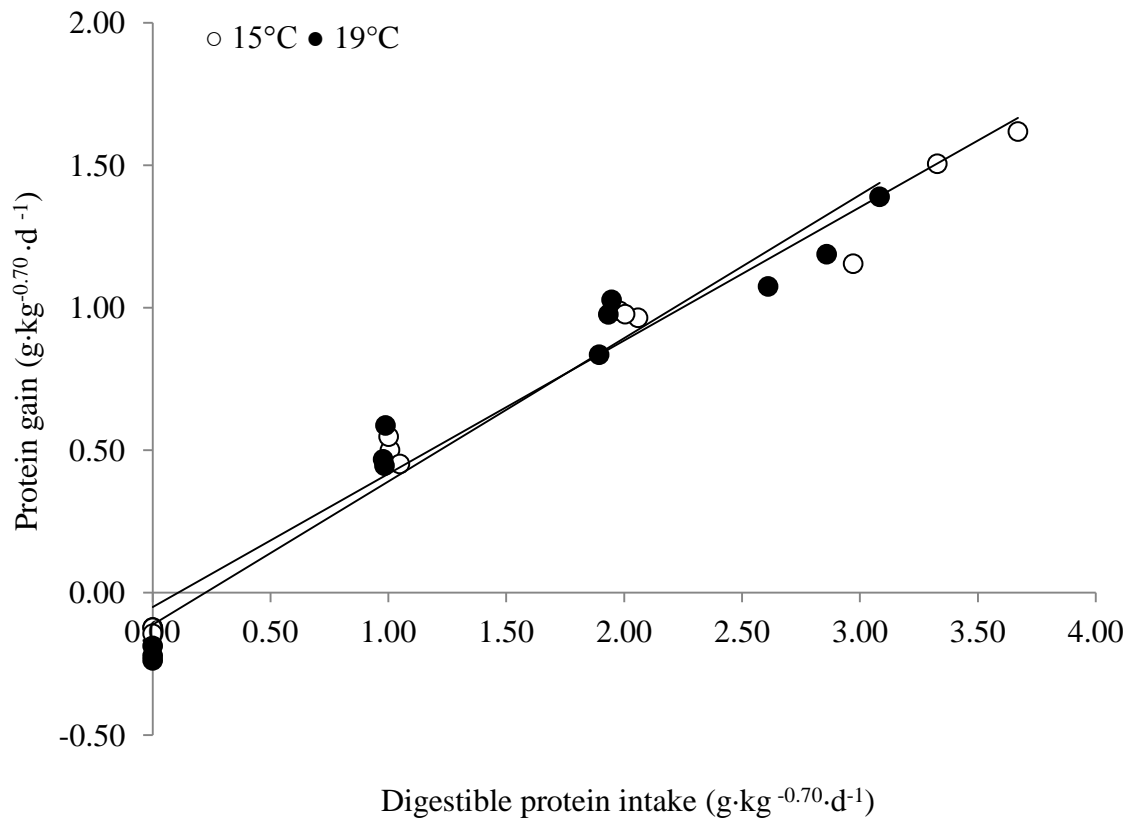


Figure 5.3. Daily protein gain per unit of metabolic weight in brook trout fed increasing amounts of digestible protein at different temperatures. Regression equations are: protein gain 15°C = $0.47 \pm 0.02\text{DPI (g}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}) - 0.050 \pm 0.05$ ($R^2=0.98$, $F_{1,10}=390.50$, $P<0.001$) and protein gain 19°C = $0.50 \pm 0.04\text{DPI (g}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}) - 0.11 \pm 0.06$ ($R^2=0.95$, $F_{1,10}=211.72$, $P<0.001$). Protein requirements for maintenance are: DP_{maint} at 15°C = $0.11 \text{ gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$ and DP_{maint} at 19°C = $0.22 \text{ gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$.

The energy gain ($\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$) against digestible energy intake (DEI) as expressed metabolic body weight can be described by the following equation (Fig. 5.4):

$$\begin{aligned} \text{Energy gain at } 15^{\circ}\text{C} (\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}) = \\ 0.63 \pm 0.02\text{DEI} (\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}) - 18.90 \pm 2.53 \quad (R^2=0.99, F_{1,10}=1043.65, P<0.001) \end{aligned} \quad (14)$$

$$\begin{aligned} \text{Energy gain at } 19^{\circ}\text{C} (\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}) = \\ 0.61 \pm 0.02\text{DEI} (\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}) - 22.24 \pm 2.26 \quad (R^2=0.99, F_{1,10}=939.31, P<0.001) \end{aligned} \quad (15)$$

Maintenance digestible energy requirement (DE_{maint}) of brook trout at both temperatures was calculated as the amount of digestible energy required (x) at zero retention of energy ($y = 0$),

$$\text{DE}_{\text{maint}} \text{ at } 15^{\circ}\text{C} = 29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} \quad (16)$$

$$\text{DE}_{\text{maint}} \text{ at } 19^{\circ}\text{C} = 36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} \quad (17)$$

The slope of linear regression shows the partial efficiency of digestible energy (K_{DE}), which was 0.63 and 0.61 at 15°C and 19°C , respectively. The reciprocal value ($1/K_{\text{DE}}$) determines the cost of digestible energy (c) to deposit per unit of energy, which was 1.58 and 1.65 at 15°C and 19°C , respectively.

Thus, the daily energy requirement (DE_{total}) for brook trout is:

$$\text{DE}_{\text{total}} \text{ at } 15^{\circ}\text{C} = 29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} + 1.58 \times \text{energy gain} \quad (18)$$

$$\text{DE}_{\text{total}} \text{ at } 19^{\circ}\text{C} = 36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1} + 1.65 \times \text{energy gain} \quad (19)$$

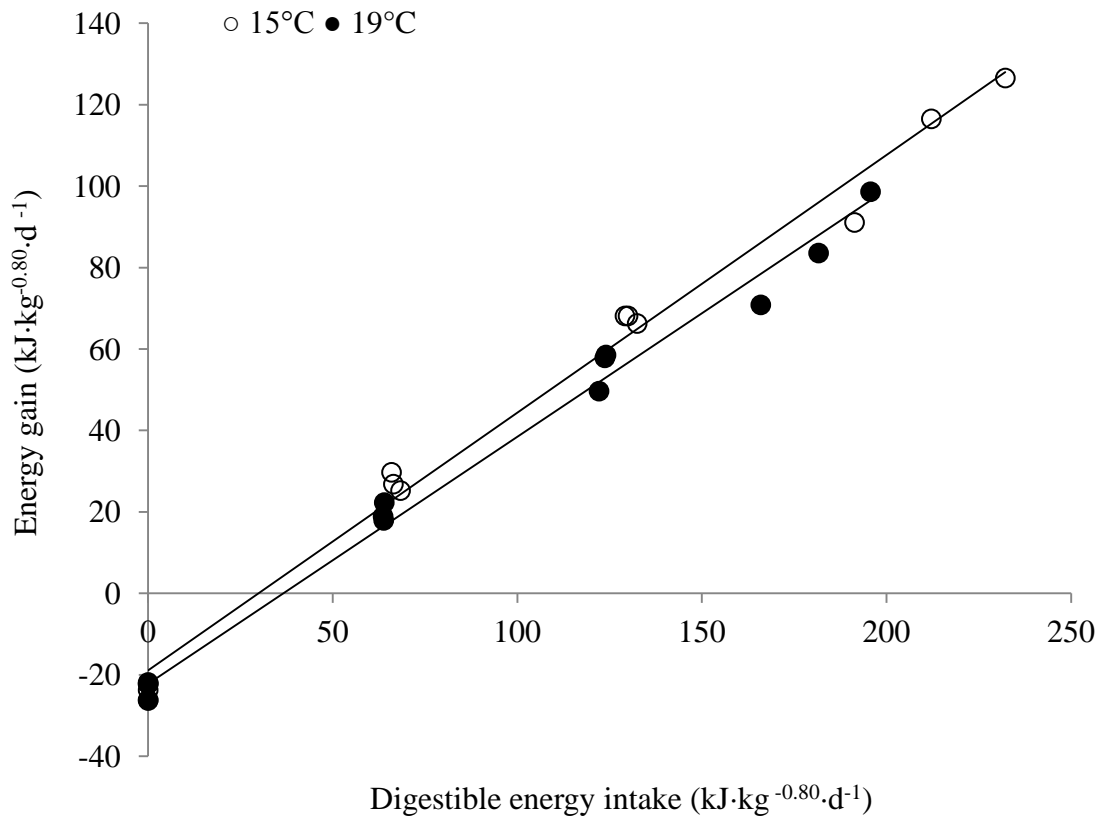


Figure 5.4. Daily energy gain per unit of metabolic weight in brook trout fed increasing amounts of digestible energy at different temperatures. Regression equations are: energy gain at 15°C = $0.63 \pm 0.02\text{DEI (kJ}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}) - 18.90 \pm 2.53$ ($R^2=0.99$, $F_{1,10}=1043.65$, $P<0.001$) and energy gain at 19°C = $0.61 \pm 0.02\text{DEI (kJ}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}) - 22.24 \pm 2.26$ ($R^2=0.99$, $F_{1,10}=939.31$, $P<0.001$). Energy requirements for maintenance are: DE_{maint} at 15°C = $29.87 \text{ kJDE}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$ and DE_{maint} at 19°C = $36.66 \text{ kJDE}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$.

5.4.4. Whole –body chemical composition

Body compositions of brook trout across the growth cycle (Fig. 5.5) were described by allometric function of fish live-weight:

$$\text{Energy (kJ}\cdot\text{g}^{-1}) = 4.89 \pm 0.25 \text{ BW(g)}^{0.09 \pm 0.01} (R^2=0.78, F_{1,16}=56.31, P<0.001) \quad (20)$$

$$\text{Protein (\%)} = 0.003 \pm 0.001 \text{ BW(g)} + 16.19 \pm 0.13 (R^2=0.61, F_{1,16}=24.62, P<0.001) \quad (21)$$

$$\text{Lipid (\%)} = 4.28 \pm 0.38 \text{ BW(g)}^{0.18 \pm 0.02} (R^2=0.84, F_{1,16}=81.09, P<0.001) \quad (22)$$

$$\text{Moisture (\%)} = 79.38 \pm 1.08 \text{ BW(g)}^{-0.02 \pm 0.003} (R^2=0.74, F_{1,16}=44.62, P<0.001) \quad (23)$$

5.4.5. Growth model

The daily growth rate (Fig. 5.6) of brook trout obtained from commercial farm dependent upon the fish body weight (BW) which was expressed by the following allometric equation:

$$\begin{aligned} \text{Daily growth rate (g}\cdot\text{fish}^{-1}\cdot\text{d}^{-1}) = \\ 0.054 \pm 0.004 \text{ BW (g)}^{0.85 \pm 0.02} (R^2=0.95, F_{1,74}=1368.33, P<0.001) \end{aligned} \quad (24)$$

5.4.6. Study output

The output of this model, the feed specification and feeding regimes for brook trout were presented theoretically in a chart for 15°C and 19°C (Table 5.2 & 5.3). The several strategies were developed (Fig. 5.7, 5.8 and 5.9) based on the table 5.2 and table 5.3.

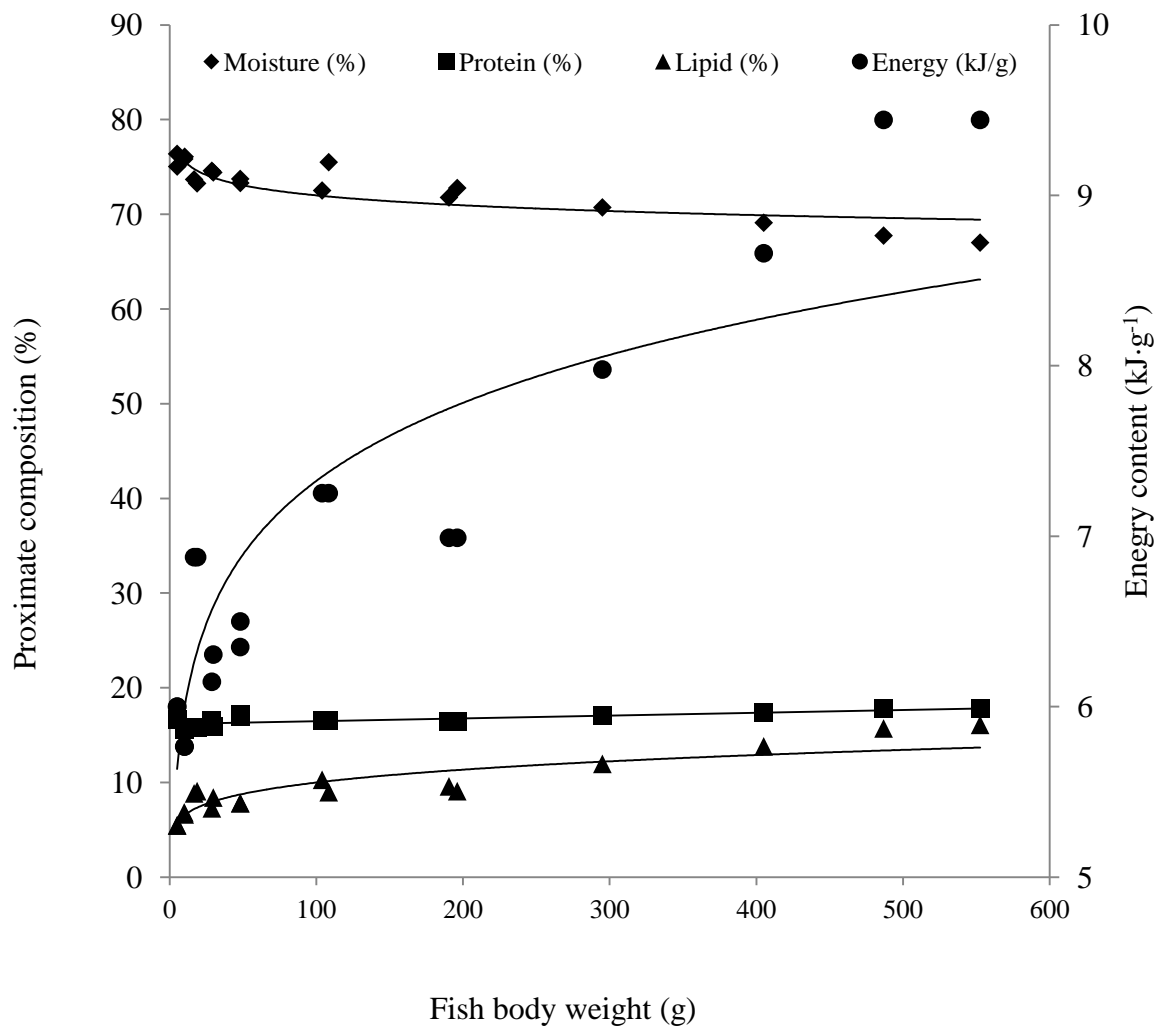


Figure 5.5. Proximate composition of brook trout at different sizes. Regression equations are: energy ($\text{kJ}\cdot\text{g}^{-1}$) = 4.89 ± 0.25 BW(g) $^{0.09 \pm 0.01}$ ($R^2=0.78$, $F_{1,16}=56.31$, $P<0.001$), protein (%) = 0.003 ± 0.001 BW(g) + 16.19 ± 0.13 ($R^2=0.61$, $F_{1,16}=24.62$, $P<0.001$), lipid (%) = 4.28 ± 0.38 BW(g) $^{0.18 \pm 0.02}$ ($R^2=0.84$, $F_{1,16}=81.09$, $P<0.001$) and moisture (%) = 79.38 ± 1.08 BW(g) $^{-0.02 \pm 0.003}$ ($R^2=0.74$, $F_{1,16}=44.62$, $P<0.001$).

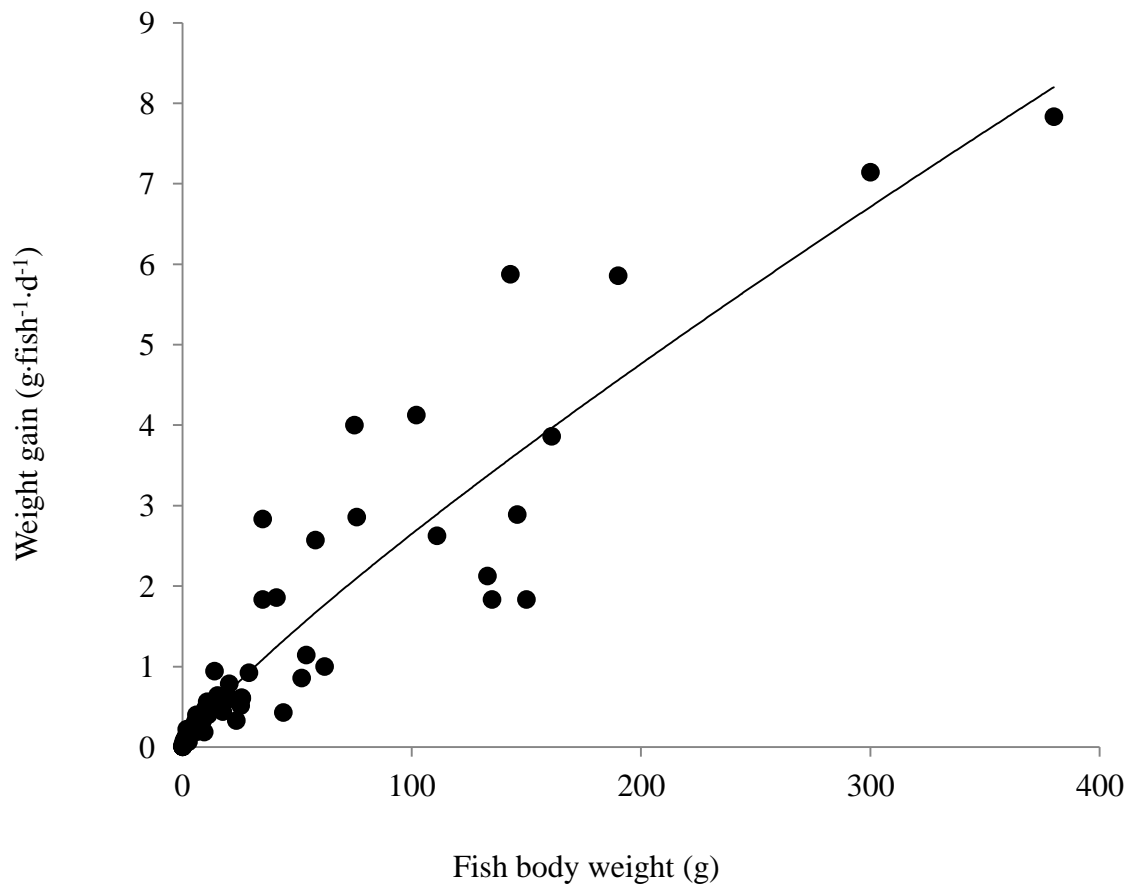


Figure 5.6. Growth rates of brook trout at different live weight size. Data was collected from a commercial farm in Tasmania. The regression equation is: weight gain (g·fish⁻¹·d⁻¹) = $0.054 \pm 0.004BW (g)^{0.85 \pm 0.02}$ ($R^2=0.95$, $F_{1,74}=1368.33$, $P<0.001$).

Table 5.2. Calculation of protein and energy requirement of brook trout and the recommended diet specification at 15°C.

Fish body weight (g fish ⁻¹)			5			10			50			100			250			500		
Growth rate (g fish ⁻¹ ·d ⁻¹)			0.21			0.38			1.47			2.65			5.75			10.34		
Energy requirement																				
Metabolic BW (kg ^{0.80})			0.014			0.025			0.091			0.158			0.330			0.574		
DE _{maint} (kJ·fish ⁻¹ ·d ⁻¹) ¹			0.43			0.75			2.72			4.73			9.85			17.16		
Energy gain (kJ·fish ⁻¹ ·d ⁻¹) ²			1.18			2.26			10.14			19.39			45.63			87.21		
DE _{growth} (kJ·fish ⁻¹ ·d ⁻¹) ³			1.86			3.56			16.03			30.63			72.10			137.79		
DE _{total} (kJ·fish ⁻¹ ·d ⁻¹) ⁴			2.30			4.31			18.75			35.36			81.96			154.94		
Protein requirement																				
Metabolic BW(kg ^{0.70})			0.025			0.040			0.123			0.200			0.379			0.616		
DP _{maint} (g·fish ⁻¹ ·d ⁻¹) ⁵			0.003			0.004			0.014			0.022			0.042			0.068		
Protein gain (g·fish ⁻¹ ·d ⁻¹) ⁶			0.033			0.061			0.240			0.437			0.974			1.830		
DP _{growth} (g·fish ⁻¹ ·d ⁻¹) ⁷			0.071			0.130			0.514			0.935			2.084			3.916		
DP _{total} (g·fish ⁻¹ ·d ⁻¹) ⁸			0.074			0.134			0.527			0.957			2.126			3.984		
Diet specification																				
Total energy content in diet		18	21	24	18	21	24	18	21	24	18	21	24	18	21	24	18	21	24	
DE in diet (MJ·kg ⁻¹) ⁹		15.74	18.36	20.98	15.74	18.36	20.98	15.74	18.36	20.98	15.74	18.36	20.98	15.74	18.36	20.98	15.74	18.36	20.98	
Feed intake (g·fish ⁻¹ ·d ⁻¹) ¹⁰		0.15	0.13	0.11	0.27	0.23	0.21	1.19	1.02	0.89	2.25	1.93	1.69	5.21	4.46	3.91	9.85	8.44	7.38	
Feed Intake (%BW·d ⁻¹)		2.92	2.50	2.19	2.74	2.35	2.06	2.38	2.04	1.79	2.25	1.93	1.69	2.08	1.79	1.56	1.97	1.69	1.48	
Expected FCR		0.70	0.60	0.52	0.73	0.62	0.55	0.81	0.69	0.61	0.85	0.73	0.64	0.91	0.78	0.68	0.95	0.82	0.71	
DP in diet (g·kg ⁻¹) ¹¹		507	591	676	489	571	652	442	516	590	426	497	568	408	476	544	405	472	540	
CP content in diet (g·kg ⁻¹) ¹²		554	647	739	535	624	713	484	564	645	466	543	621	446	521	595	442	516	590	
DP : DE ratio (g·MJ ⁻¹)		32.22	32.22	32.22	31.09	31.09	31.09	28.12	28.12	28.12	27.06	27.06	27.06	25.94	25.94	25.94	25.71	25.71	25.71	

- ¹ daily requirement of digestible energy for maintenance, calculated by using the equation 16.
- ² daily energy gain, calculated by using equation 20.
- ³ daily requirement of digestible energy for growth calculated at 0.63 partial efficiency of energy = $1.58 \times \text{prospective energy gain}$
- ⁴ the total daily requirement of energy = $DE_{\text{maint}} + DE_{\text{growth}}$
- ⁵ the daily requirement of digestible protein for maintenance, calculated by using formula 10
- ⁶ prospective protein gain, calculated by using equation 21
- ⁷ daily requirement of digestible protein for growth calculated at 0.47 partial efficiency of protein = $2.14 \times \text{prospective protein gain}$
- ⁸ the total daily requirement of protein = $DP_{\text{maint}} + DP_{\text{growth}}$
- ⁹ based on the apparent digestibility of energy
- ¹⁰ required feed intake to meet total daily digestible energy requirement by using diet (DE: $19.23 \text{ MJ} \cdot \text{kg}^{-1}$)
- ¹¹ required DP ($\text{g} \cdot \text{kg}^{-1}$) inclusion in diet to meet total daily digestible protein requirement = $(DP_{\text{total}} \cdot \text{feed intake}^{-1}) \times 1000$
- ¹² based on the apparent digestibility of protein

Table 5.3. Calculation of protein and energy requirement of brook trout and the recommended diet specification at 19°C.

Fish body weight (g fish ⁻¹)	5			10			50			100			250			500		
Growth rate (g·fish ⁻¹ ·d ⁻¹)	0.21			0.38			1.47			2.65			5.75			10.34		
<i>Energy requirement</i>																		
Metabolic BW (kg ^{0.80})	0.014			0.025			0.091			0.158			0.330			0.574		
DE _{maint} (kJ·fish ⁻¹ ·d ⁻¹) ¹	0.53			0.92			3.34			5.81			12.09			21.06		
Energy gain (kJ·fish ⁻¹ ·d ⁻¹) ²	1.18			2.26			10.14			19.39			45.63			87.21		
DE _{growth} (kJ·fish ⁻¹ ·d ⁻¹) ³	1.95			3.72			16.74			31.99			75.30			143.89		
DE _{total} (kJ·fish ⁻¹ ·d ⁻¹) ⁴	2.48			4.64			20.08			37.80			87.39			164.95		
<i>Protein requirement</i>																		
Metabolic BW(kg ^{0.70})	0.025			0.040			0.123			0.200			0.379			0.616		
DP _{maint} (g·fish ⁻¹ ·d ⁻¹) ⁵	0.005			0.009			0.027			0.044			0.083			0.135		
Protein gain (g·fish ⁻¹ ·d ⁻¹) ⁶	0.033			0.061			0.240			0.437			0.974			1.830		
DP _{growth} (g·fish ⁻¹ ·d ⁻¹) ⁷	0.066			0.121			0.478			0.870			1.938			3.642		
DP _{total} (g·fish ⁻¹ ·d ⁻¹) ⁸	0.072			0.129			0.505			0.914			2.022			3.777		
<i>Diet specification</i>																		
Total energy content in diet	18	21	24	18	21	24	18	21	24	18	21	24	18	21	24	18	21	24
DE in diet (MJ·kg ⁻¹) ⁹	15.06	17.57	20.08	15.06	17.57	20.08	15.06	17.57	20.08	15.06	17.57	20.08	15.06	17.57	20.08	15.06	17.57	20.08
Feed intake (g·fish ⁻¹ ·d ⁻¹) ¹⁰	0.16	0.14	0.12	0.31	0.26	0.23	1.33	1.14	1.00	2.51	2.15	1.88	5.80	4.97	4.35	10.95	9.39	8.22
Feed Intake (%BW·d ⁻¹)	3.29	2.82	2.47	3.08	2.64	2.31	2.67	2.29	2.00	2.51	2.15	1.88	2.32	1.99	1.74	2.19	1.88	1.64
Expected FER	0.79	0.67	0.59	0.82	0.70	0.61	0.91	0.78	0.68	0.95	0.81	0.71	1.01	0.86	0.76	1.06	0.91	0.79
DP in diet (g·kg ⁻¹) ¹¹	436	508	581	420	490	560	379	442	505	364	425	485	348	406	464	345	402	460
CP content in diet (g·kg ⁻¹) ¹²	490	572	654	473	551	630	426	497	568	410	478	546	392	457	523	388	453	517
DP : DE ratio (g·MJ ⁻¹)	28.94	28.94	28.94	27.88	27.88	27.88	25.14	25.14	25.14	24.17	24.17	24.17	23.13	23.13	23.13	22.90	22.90	22.90

- ¹ daily requirement of digestible energy for maintenance, calculated by using the equation 17.
- ² daily energy gain, calculated by using equation 20.
- ³ daily requirement of digestible energy for growth calculated at 0.61 partial efficiency of energy = $1.65 \times \text{prospective energy gain}$
- ⁴ the total daily requirement of energy = $DE_{\text{maint}} + DE_{\text{growth}}$
- ⁵ the daily requirement of digestible protein for maintenance, calculated by using formula 11
- ⁶ prospective protein gain, calculated by using equation 21
- ⁷ daily requirement of digestible protein for growth calculated at 0.50 partial efficiency of protein = $1.99 \times \text{prospective protein gain}$
- ⁸ the total daily requirement of protein = $DP_{\text{maint}} + DP_{\text{growth}}$
- ⁹ based on the apparent digestibility of energy
- ¹⁰ required feed intake to meet total daily digestible energy requirement by using diet (DE: $18.41 \text{ MJ} \cdot \text{kg}^{-1}$)
- ¹¹ required DP ($\text{g} \cdot \text{kg}^{-1}$) inclusion in diet to meet total daily digestible protein requirement = $(DP_{\text{total}} \cdot \text{feed intake}^{-1}) \times 1000$
- ¹² based on the apparent digestibility of protein

5.5. Discussion

This is the first study to determine the protein and energy requirements of brook trout by factorial approach. These requirements have been determined at 15°C, as the optimum temperature for brook trout and at 19°C, as the summer temperature. These finding can be used to develop a feeding chart in relation to winter and summer temperature in commercially brook trout farm.

Whole-body chemical composition of fish depends on the fish weight (Shearer, 1994; Dumas et al., 2010). Whole-body protein content (%) of brook trout were almost constant throughout the life cycle, which was not surprising in fish (Dumas et al., 2010). Whole-body energy ($\text{kJ}\cdot\text{g}^{-1}$) and lipid (%) content of brook trout increased with its size and the moisture content was inversely related to lipid content, which is typical in fish (Shearer, 1994; Dumas et al., 2007).

5.5.1. Protein requirement and efficiency

There is some discussion regarding the effect of temperature on the maintenance protein requirement of fish (Carter et al., 2008; Lupatsch, 2009; Pirozzi et al., 2010b). In mulloway, *Argyrosomus japonicas*, the protein requirement for maintenance was independent to temperature (Pirozzi et al., 2010b), however, temperature effects have been determined in some fish species (Carter et al., 2008; Lupatsch, 2009). This study showed that the daily maintenance protein requirement of brook trout was dependent upon water temperature, which was $0.11 \text{ gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$ at 15°C and $0.22 \text{ gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$ at 19°C. Maintenance protein requirement ($\text{gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$) of Atlantic salmon ranged from 0.118 at 10°C (Helland et al., 2010) to 0.50 at 12°C to 0.82 at 19°C (Carter et al., 2008). The noticeable difference among the studies may be due to the experimental condition and technique that used to calculate the requirement. The maintenance protein requirement ($\text{gDP}\cdot\text{kg}^{-0.70}\cdot\text{d}^{-1}$) of Atlantic salmon was 4.58 using ration-growth curve and it was 0.82 using comparative slaughter technique. The maintenance protein requirement of rainbow trout was $1.1 \text{ gDP}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ at 15°C (Storebakken et al., 1991).

The partial retention of efficiency of protein (K_{DP}) was not affected by temperature (Lupatsch, 2009). The K_{DP} in brook trout was 0.47 and 0.50 at 15°C and 19°C, respectively, which was similar to gilthead sea bream (K_{DP} = 0.47; Lupatsch, 2009) and rainbow trout (K_{DP} = 0.60; Glencross et al., 2008b; K_{DP} = 0.41; Glencross et al., 2008a). Brook trout have higher protein efficiency than beluga sturgeon (K_{DP} = 0.38; Amrkolaie et al., 2012). In contrast, a higher K_{DP} of 0.64 was found in Atlantic salmon reared at 10°C in saltwater (Helland et al., 2010), however, K_{DP} was close 0.47 in fish fed fish meal based diet which generally contained a balanced amino acid profile (Lupatsch, 2009). The K_{DP} was increased when the dietary crude protein level decreased, as a result of a lower proportion of dietary protein being utilised for energy (Lupatsch et al., 2001b; Hatlen et al., 2007). Although only one feed was used in this study this principle explain the slightly higher K_{DP} at 19°C due to poorer digestibility of protein, which made less protein available for energy. A slightly higher K_{DP} of 0.48 was found in gilthead sea bream at 27°C than that of 0.46 at 20°C or 24°C (Lupatsch, 2009).

5.5.2. *The energy requirement and efficiency*

The maintenance energy requirement of fish depends on the temperature (Carter et al., 2008; Lupatsch, 2009; Pirozzi et al., 2010b). At high temperatures the energy requirement is increased for higher metabolic rate (Jobling, 1997; Katersky and Carter, 2007). The daily maintenance energy requirement of brook trout was 29.87 kJ kg^{-0.80} at 15°C and 36.66 kJ kg^{-0.80} at 19°C. The daily maintenance energy requirement in rainbow trout was 19 kJ kg^{-0.88} at 8.5°C (Bureau et al., 2006), 41 kJ kg⁻¹ at 15°C (Storebakken et al., 1991), in Atlantic salmon 31.5 kJ kg^{-0.8} at 10°C (Helland et al., 2010), in gilthead sea bream 47.89 kJ kg^{-0.80} at 23°C, European sea bass 45.38 kJ kg^{-0.80} at 23°C and white grouper 34.05 kJ kg^{-0.80} at 23°C (Lupatsch et al., 2003). It was suggested that the maintenance energy requirement of fish was between 40- 60 kJ kg^{-0.80} d⁻¹ at their optimum rearing temperature (Bureau et al., 2002). In juvenile mullet, the maintenance energy requirements were increased from 44.2 kJ to 49.6 kJ kg^{-0.8} d⁻¹ when the rearing increased from 20°C to 26°C (Pirozzi et al., 2010b).

The energy efficiency for growth (K_{DE}) in fish was not affected by fish dietary energy level and temperature, but species differences were evident (Azevedo et al., 1998;

Hatlen et al., 2007; Lupatsch, 2009; Helland et al., 2010). The K_{DE} found in this study was similar to previous studies in rainbow trout, where the K_{DE} was 0.61 regardless of the feeding level or temperature (Azevedo et al., 1998), 0.63 (Bureau et al., 2006) and 0.68 (Rodehutsord and Pfeffer, 1999). In contrast to this, a higher $K_{DE} = 0.74$ was found in rainbow trout fed lupin kernel meal based diet (Glencross et al., 2008b). The K_{DE} in brook trout found in this study were less than that found in Atlantic salmon, which was 0.80 (Helland et al., 2010). The differences among the studies were possibly due to dietary effects on K_{DE} , which was observed in grass carp (Carter and Brafield, 1991). Another possible reason for this difference in K_{DE} may be explained by the fish size used in the experiment. The K_{DE} in barramundi was 0.61 for 15 g fish, which was increased to 0.76 for 410 g fish (Glencross, 2008). The noticeable differences in energy efficiency in relation to size may be important to further develop factorial modelling and feed formulation for brook trout, and deserves further research.

5.5.3. Scope of application

According to the available literature, optimal growth of brook trout was around 15°C. The growth data obtained from a commercial farm in Tasmania, Australia, where the average summer temperatures often reached at 19-20°C, have been used to develop the theoretical feeding chart. As commercial farms do not operate under temperature control, the growth rate of the complete production cycle at single temperature was not available. Thus, common growth data were used for both temperatures in this study, which may not explain the potential effect of temperature on the dietary specification. Another potential consideration is that growth data obtained from different farms may vary and diet specification can be recalculated by using these models.

Based on tables 2 and 3, there were several strategies might be obtained to calculate the dietary composition for brook trout based on the energy density of diet and size of the fish. Digestible protein to energy requirement (DP:DE, g·MJ⁻¹) was dependent on the fish weight (g). The equation (Fig. 5.7) derived from this relationship were, DP: DE, g MJ⁻¹ = 34.82 BW(g)^{-0.05} at 15 °C ($R^2 = 0.98$) and DP: DE, g·MJ⁻¹ = 31.36 BW(g)^{-0.05} at 19°C ($R^2 = 0.98$) can be used to determine the digestible protein to energy requirement of brook trout.

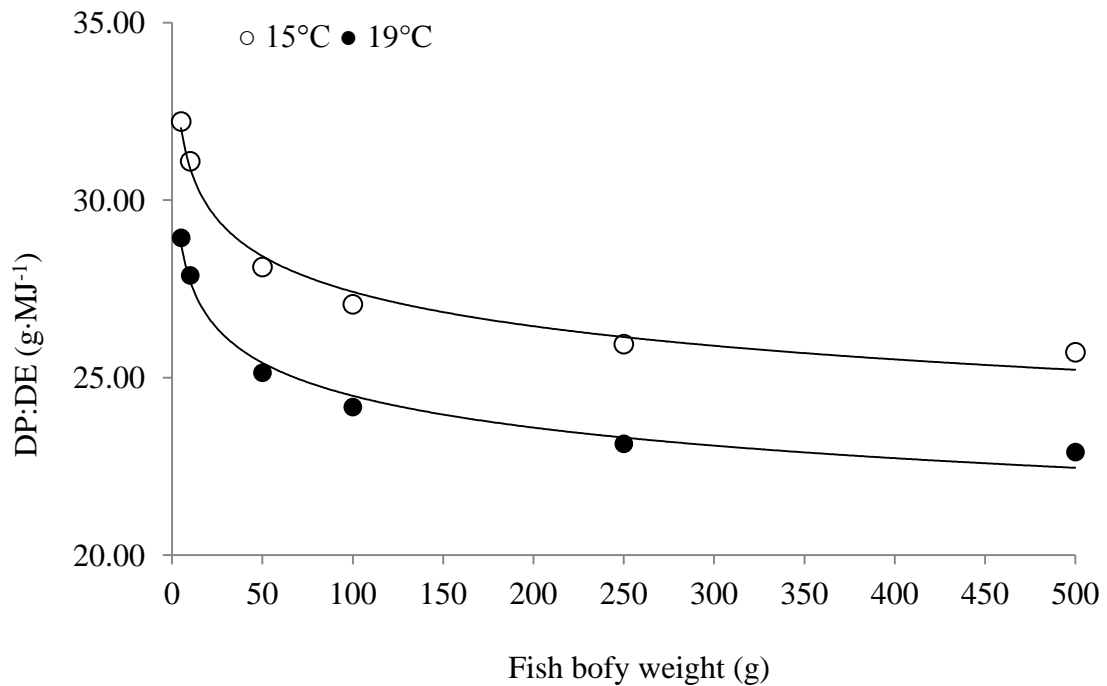


Figure 5.7. The theoretical requirement for dietary protein to energy ratio (DP:DE, g·MJ⁻¹) at the different sizes for brook trout at two temperatures (15°C and 19°C). The allometric equation were: DP: DE, g·MJ⁻¹ = 34.82 BW(g)^{-0.05} at 15 °C ($R^2 = 0.98$) and DP: DE, g·MJ⁻¹ = 31.36 BW(g)^{-0.05} at 19°C ($R^2 = 0.98$).

The selection of energy densities affected the theoretical feed intake ($\%BW \cdot d^{-1}$), FCR and dietary protein content for fish (Table 5.2 & 5.3). Lower FCR can be achieved in brook trout fed with high energetic diet (Table 5.2 & 5.3; Fig. 5.8), which was common in either fish or crustacean (Ward et al., 2003; Pirozzi et al., 2010a; Glencross et al., 2011). In this case, high dietary content of protein were needed to meet the requirement (Table 5.2 & 5.3; Fig. 5.8), however, it does not necessarily mean that high protein diet always increase the efficiency. In case of well balanced diet, growth efficiency and protein retention will be high as the protein synthesis is high (Carter et al., 2008). Even poor diet may stimulate high growth and protein synthesis if the protein degradation is low (Carter et al., 2008). When protein intake was limiting, non-protein energy intake is increased to meet the total energy requirement (Kaushik and Médale, 1994). Thus, aquafeeds should contain substantial amount of digestible non-protein energy.

Moreover, there were tendencies to increase the FCR (low growth efficiency) with increasing the body weight of fish (Fig. 5.8). It is not surprising that matured fish have less growth efficiency than juvenile fish. Juvenile fish require less energy per unit weight gain and have high rate of protein synthesis and retention (Kaushik and Médale, 1994). In juvenile fish, up to 40% of total energy is used for protein synthesis (Carter and Houlihan, 2001). This is not problematic if the growth is high and protein is retained as growth (Carter et al., 2008).

Feed intake ($\%BW \cdot d^{-1}$) was lower at juvenile brook trout and lower feed intake can be achieved fed with high energy diet (Fig. 5.9). It is well accepted that increase in energy content of diet reduces the feed intake. Fish consume feed to meet their energy requirement (Jobling, 1994; Kaushik and Médale, 1994). Thus the rationale of feeding to fish is to supply surplus energy to meet their requirement under given farming condition. Considering this point, feeding charts for brook trout depending on the daily nutrient requirement, size, growth rate and water temperature have been developed.

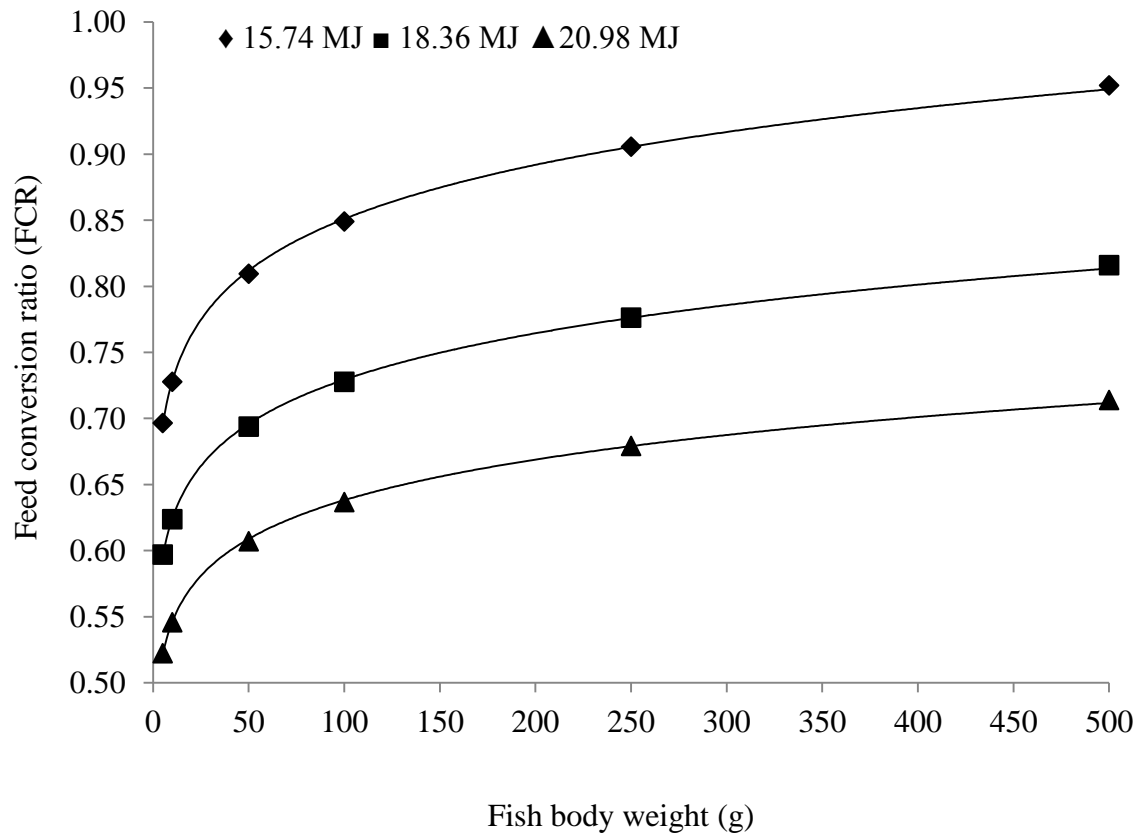


Figure 5.8. The theoretical FCR at different sizes of brook trout fed diets having three different digestible energy densities (15.74, 18.36 and 20.98 MJ·kg⁻¹). The allometric equation are $FCR = 0.62BW(g)^{0.07}$ ($r^2 = 1.00$), $FCR = 0.53 BW(g)^{0.07}$ ($R^2 = 1.00$) and $FCR = 0.47 BW(g)^{0.07}$ ($R^2 = 1.00$), respectively. FCR increased with increasing weight of fish at 15°C, similar trend was found at 19°C (not shown in figure).

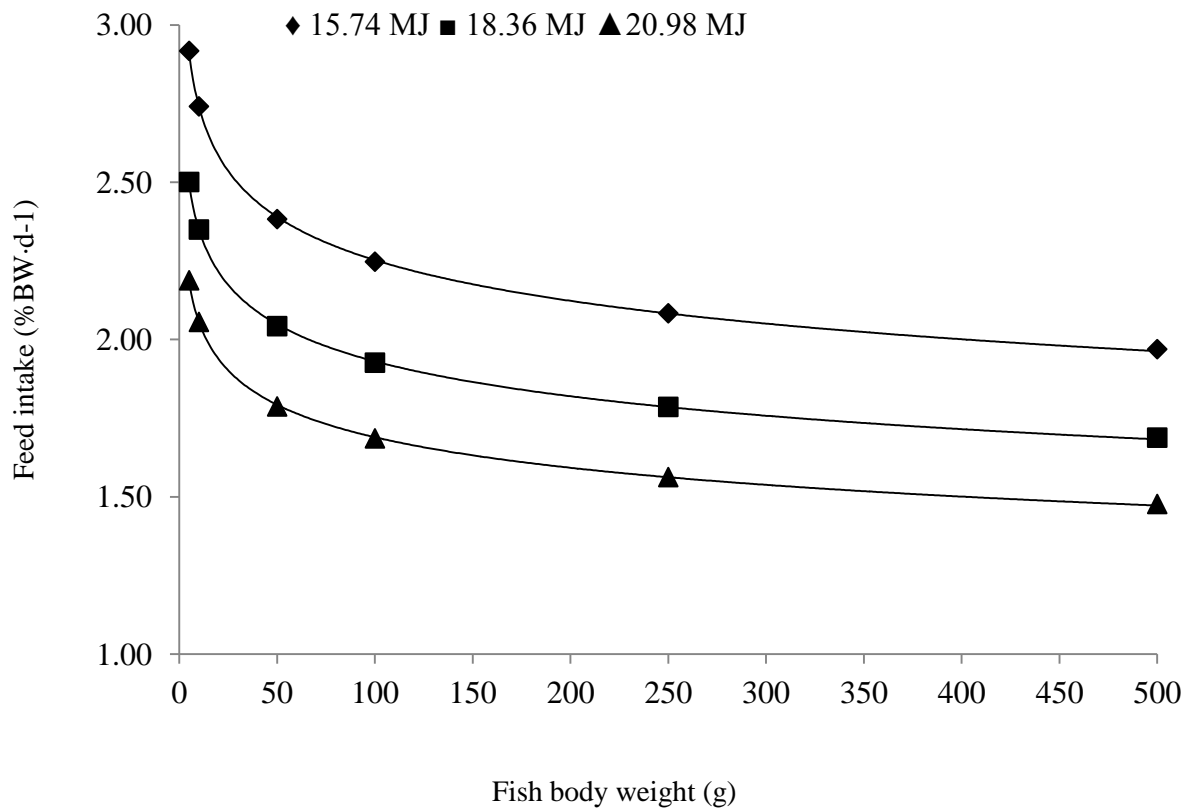


Figure 5.9. The relationship between feed intake (FI) and different sizes of brook trout fed diets having three different digestible energy densities (15.74, 18.36 and 20.98 MJ·kg⁻¹). The allometric equation are: $FI = 3.34 BW(g)^{-0.09}$ ($R^2 = 1.00$), $FI = 2.86 BW(g)^{-0.09}$ ($R^2 = 1.00$) and $FI = 2.50 BW(g)^{-0.09}$ ($R^2 = 1.00$), respectively. FI decreased with increasing weight of fish at 15°C, similar trend was found at 19°C (not shown in figure).

5.6. Conclusion

Both the protein and energy requirement for maintenance in brook trout were dependent on the rearing temperature of fish. Compared to 15°C, an almost doubling amount of protein would be required to meet the protein demand for maintenance by brook trout at 19°C. Higher protein demand at high summer temperatures indicated that protein was also catabolised as an energy source to meet the increased energy demand in this fish. The models obtained from this study may need to be recalculated if new protein sources are used to replace the fish meal. It is well accepted protein sources from different plant ingredient are not equally utilised by fish.

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CHAPTER 6

GENERAL DISCUSSION

6.1. Overview of thesis

This thesis has defined protein and energy requirement on brook trout, *Salvelinus fontinalis*, in relation to water temperature. According to the available literature, the optimum temperature for brook trout is 15°C and a number studies have investigated the effect of temperature on the growth of brook trout at or below the optimum temperature (Baldwin, 1957; Gunther et al., 2007; Fischer et al., 2009). However, in Tasmania, brook trout are normally farmed at temperatures approaching 20°C (Miller et al., 2006; Ng et al., 2010). Detailed studies to determine protein and energy requirements of brook trout, nutrient utilisation, nutrient digestibility and growth performance were studied at 15°C and 19°C. Key findings of experimental chapters (2-5) are:

Chapter 2:

- At both temperatures, the dietary crude protein requirement was 44%.
- Apparent digestibility of protein (AD_{CP}) was higher at 15°C than at 19°C.
- Higher growth rate of brook trout was obtained when fish were reared at 15°C compared to 19°C.
- Supplementing dietary energy with pre-gelatinised maize starch supported good growth performance and increased protein efficiency for growth in brook trout at both temperatures.
- Increasing levels of dietary carbohydrate increased lipid deposition in fish.

Chapter 3:

- The pyruvate kinase (PK) activity was higher at 19°C than at 15°C indicating that brook trout have a greater capacity to use gelatinised maize starch at high temperature.
- Higher levels of gelatinised starch increased the PK activity and also reduced GDH activity. The utilisation of gelatinised carbohydrate through glycolysis indicated by PK activity was stimulated by increased dietary gelatinised carbohydrate inclusion.
- In addition, reduction of protein catabolism (indicated by decreased GDH activity) was stimulated by increasing levels of dietary gelatinised carbohydrate

inclusion and at least 13% dietary gelatinised carbohydrate should be added to brook trout feeds to reduce protein catabolism

- Lipogenic enzyme G6PDH was not affected by increasing dietary gelatinised carbohydrate indicating that lipid synthesis via the HMP shunt was not induced by increasing content of gelatinised carbohydrate. Lipid synthesis might have occurred from acetyl-CoA originating from glycolysis of carbohydrate (increasing PK activity).
- Gelatinised maize starch can be used as good sources of non protein energy in the diet of brook trout.

Chapter 4:

- Gelatinised carbohydrate (18-26%) successfully replaced lipid (17-13%) energy without any adverse effects on nutrient digestibility, growth, liver and gut structures in brook trout.
- Apparent digestibility of dry matter, protein, lipid and energy was significantly higher at 15°C than at 19°C.
- In iso-nitrogenous and iso-energetic diets, higher levels of gelatinised carbohydrate improved the apparent digestibility of dry matter, gross energy and energy from carbohydrate.

Chapter 5:

- The protein and energy requirement for maintenance and growth in brook trout was higher at 19°C than at 15°C.
- The maintenance requirement of protein was $0.11 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ and $0.22 \text{ gDP} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ at 15°C and 19°C, respectively.
- The maintenance energy requirement was $29.87 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ and $36.66 \text{ kJDE} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ at 15°C and 19°C, respectively.

6.2. Carbohydrate utilisation on brook trout and salmonid

The first study conducted by Phillips et al. (1948) on the utilisation of carbohydrate by salmonids concluded that trout diets should contain less than 12% carbohydrate. Diets which contained higher levels of carbohydrate increased mortality and reduced growth

(Phillips et al., 1948). Rainbow trout were unable to utilise dietary carbohydrate over 14% (Hilton and Atkinson, 1982). In contrast, increasing dietary carbohydrate from 15 to 30% did not show any adverse effect on growth and feed utilisation of rainbow trout (Bergot, 1979). A diet containing 9% carbohydrate was optimal for Atlantic salmon (Hemre et al., 1995). The optimum level of carbohydrate of Atlantic salmon, based on the feed efficiency, growth and muscle pigmentation was determined to be 10% (Aksnes, 1995). In contrast, dietary carbohydrate levels up to 21% did not have a negative effect on growth per g of dietary protein of Atlantic salmon (Hillestad et al., 2001). Due to poorer digestion capability, and technical processing considerations crude starch content in commercial salmon feeds have a 20% upper inclusion limit (Pieper and Pfeffer, 1980; Spannhof and Plantikow, 1983). Using higher levels gelatinised carbohydrate to improve protein and energy utilisation, the level has exceeded 30% in rainbow trout (Kaushik et al., 1989; Kim and Kaushik, 1992). Similarly, a diet containing 18-27% of gelatinised starch showed higher feed efficiency in rainbow trout (Yamamoto et al., 2001). Up to 26% gelatinised maize starch was used in this study on brook trout and did not show any adverse effect on nutrient digestibility, protein and energy efficiency and gut or liver histology. Although upper threshold has not been concluded in this study, it can be suggested that brook trout are capable of using gelatinised carbohydrate like other trout. The level of carbohydrate (15.5 to 17.5%) is being used in commercial trout aquafeeds could be increased.

6.3. Nutrition at high temperature

Many aquaculture species are currently exposed to increasing water temperature and the general trend is predicted to continue (Pittock, 2003; Lorentzen, 2008; Lough and Hobday, 2011; FAO/NACA, 2012). Salmonid culture in Australia is approaching to the upper thermal tolerance limit for species (Pankhurst and King, 2010; Barnes et al., 2011; Hobday and Lough, 2011; De Silva, 2012). All cultured aquatic species are poikilothermic and increasing water temperatures cause an exponential increase in metabolic rate, this combined with decreasing oxygen solubility result in negative effects on growth performance (Jobling, 1997; Katersky et al., 2006; Barnes et al., 2011). Since fish consume to meet their energy requirement, feeding fish at elevated temperatures to satiation means fish will consume enough food to meet their metabolic demand, feed intake will increase until appetite is inhibited (Kaushik and Médale, 1994;

Jobling, 1997; Battaglene et al., 2008). When ration is restricted, the scope of growth is progressively reduced at high temperatures because more energy is allocated for maintenance metabolism (Jobling, 1994). Satiation feeding at high temperature is assumed to be beneficial; however, unlimited supply of food is not practical to improve the growth and feed efficiency at high temperatures due to the effects of increased oxygen demand with feeding (Jobling, 1994; Jobling, 1997; Katersky et al., 2006). The benefit of satiation feeding diminished at high temperatures in coral-reef fish, *Acanthochromis polyacanthus* and growth rate was not significantly increased compared to low temperatures (Munday et al., 2008). The growth and feed efficiency of brook trout was lower at 19°C than at 15°C which is consistent to Atlantic salmon, where growth rate and feed conversion efficiency (FCE) was lower at 18°C than at 14°C (Chapter 2, 4, 5; Handeland et al., 2008).

Within the thermal tolerance range, metabolic rate (energy losses) is increased with temperature and nutrient ingestion rate (energy intake) is also increased and peak at the optimal temperature (Jobling, 1997). The difference between the rate of energy intake and the rate of energy loss represents the resources available for growth (Jobling, 1994; Jobling, 1997). In addition, high temperatures reduce dissolve oxygen (DO) level, which limit the growth potential at high temperature due to as inability of the respiratory system to provide oxygen to respiring tissues under high oxygen demand for increased metabolism (Jobling, 1997; Katersky and Carter, 2007a). The oxygen demand ($\text{O}_2\text{-fish}^{-1}\cdot\text{h}^{-1}$) of fish is increased during and shortly after feeding, known as specific dynamic action (SDA), which is approximately double the routine metabolism and remains elevated for long periods of time, depending on water temperature, meal size and composition (Jobling, 1994; Carter and Houlihan, 2001; Katersky et al., 2006). Consequently, increased SDA accelerates the stress of fish at high temperature and limits the ability of fish to consume feed (Katersky and Carter, 2007a) and this may be the cause of lowered feed intake of brook trout at high temperature in this study. Energy required for increased SDA is also obtained from digestion and absorption of nutrient and protein catabolism (Jobling, 1994). The lower growth potential of fish at high temperature is a cumulative effect of increased energy expenditure for increased metabolism, reduced feed intake and decreased oxygen availability (Jobling, 1997; Koskela et al., 1997; Katersky and Carter, 2007a).

Since the more nutrients are catabolised for increased metabolism at high temperature, the whole-body chemical composition of fish is affected (Jobling, 1994; Koskela et al., 1997; Bendiksen et al., 2003; Katersky and Carter, 2005). When fish are fed, they grow and the supplied nutrients are deposited in the body as protein and lipid. The rate of protein deposition is the difference between the rate of protein synthesis and the rate of protein degradation, is termed as protein turnover (Jobling, 1994). Protein turnover occurs when protein synthesis exceeds protein degradation and it is evident that the rate of protein synthesis is maximised at the optimum temperature of any fish species (McCafferty and Houlihan, 1997). Higher levels of whole-body protein at high temperature was found carp *Labeo rohita* (Kumar et al., 2012), however, when temperature exceeded the optimal temperature whole-body protein level is decreased (Katersky and Carter, 2005). The lower whole-body protein in brook trout at 19°C compared to 15°C was possibly due to lower protein turnover and increased metabolism at high temperature (Jobling, 1994; Katersky and Carter, 2005; Katersky and Carter, 2007b). Whole-body lipid in brook trout was higher at 15°C than at 19°C and was similar to previous studies where whole-body lipid levels of fish peaked at the optimal temperature and then decreased at higher temperatures (Koskela et al., 1997; Katersky and Carter, 2005). A similar trend in whole-body energy content of brook trout was also evident as seen in previous studies (Koskela et al., 1997; Katersky and Carter, 2005), was likely a result of increased energy demand at high temperature (Jobling, 1997). Brook trout required more energy at high temperature (chapter 5).

Protein and lipid are used as energy sources in carnivorous fish, which are obtained from fish meal and fish oil, respectively. Expansion of the aquaculture industry in areas where temperatures approach the upper thermal tolerance level for a given species means an increasing demand of wild harvested fish to provide protein and oil (Hobday et al., 2008). In spite of the increasing demand, the production of fish meal and fish oil remains static; and their price has increased annually (FAO, 2012). In order to produce cost effective aquafeeds at elevated temperatures, alternative energy sources from either plant protein and oil or carbohydrate need to be added and balanced.

In all experiments in this study, nutrient digestibility, including carbohydrate energy was lower at high temperature. Lower apparent nutrient digestibility at higher temperatures in brook trout may be caused by faster gastric evacuation rates (He and

Wurtsbaugh, 1993; Sweka et al., 2004; Pérez-Casanova et al., 2009). Temperature accounted for 72-91% of the variation of gastric evacuation rate in fish (Garcia and Adelman, 1985). Gastric evacuation rate of brook trout was estimated at 4°C to 17°C and it was increased with increasing temperature (Sweka et al., 2004). However, in this study when the dietary lipid level decreased, the digestibility of carbohydrate energy increased at both temperatures (15°C or 19°C) (Chapter 4). Higher levels of dietary lipid had a negative effect on nutrient digestibility as well as carbohydrate digestibility in Atlantic halibut (Berge and Storebakken, 1991) and ruminant animals (Doreau and Chilliard, 1997). Considering these findings, aquafeeds with higher carbohydrate energy rather than lipid energy may be a better choice of energy source at high temperature.

Some enzymes associated with energy production from carbohydrate show temperature compensation (Couto et al., 2008; Enes et al., 2008b; Moreira et al., 2008). The breakdown of dietary carbohydrate for energy production means carbohydrate as glucose is converted to pyruvate through glycolysis and subsequently it is broken down to energy through Krebs cycle (Rawles et al., 2008). The activity of enzymes involved with glycolysis were increased with increasing temperature in fish indicated that carbohydrate was used as an energy source to fulfil the energy requirement (Hilton et al., 1982; Couto et al., 2008; Enes et al., 2008b; Enes et al., 2008a). Pyruvate kinase activity was higher at elevated temperature indicating that carbohydrate utilisation was higher at 19°C than 15°C (Chapter 3). This was also evident in rainbow trout where more dietary carbohydrate was utilised for energy at 18°C in comparison to 8°C (Brauge et al., 1995).

6.4. Model validation for protein requirement

Three macronutrients, protein, lipid and carbohydrate are the energy sources in fish diets. The dose-response requirement model is useful to determine the confounding effect of all three macronutrient that produce satisfactory growth of fish. When protein was replaced by carbohydrate in iso-caloric diets, the growth of salmon per g of digestible protein increased with decreasing dietary protein level and suggested that carbohydrate saved protein for growth (Hillestad et al., 2001). A diet containing 40% protein with either 18% lipid and 18% carbohydrate or 11% lipid and 27% carbohydrate was optimal for rainbow trout (Yamamoto et al., 2001). The protein requirement of sharpsnout

seabream, *Diplodus puntazzo* was 45% when the diet contained either 30% carbohydrate and 12% lipid or 25% carbohydrate and 18% lipid (Coutinho et al., 2012). The protein requirement of this species increased to 63% when the diet contained 19% lipid and 18% carbohydrate (Vivas et al., 2006). From these two findings, it has been clearly seen that availability of non-protein energy and their combination has reduced the protein requirement of fish. Thus, combinations of macronutrient to optimise protein requirement is necessary (Grisdale-Helland and Helland, 1997) and it can be expressed using the dose-response model.

The protein requirement of Atlantic halibut was 58%, 41% and 35% for 30-100g, 560 g and 970 g fish, respectively (Árnason et al., 2009). Lower protein requirement of fish can be obtained by feeding highly digestible protein with balanced amino acid profile and adequate digestible protein to energy (Oliva-Teles, 2012). In addition, crude protein requirement of fish was also temperature dependant. At high temperature, fish require more protein and energy to maintain their higher metabolic demand. Thus, determination of crude protein requirement across the life-cycle of fish in relation to variety of environmental factors and dietary conditions is time consuming. For this purpose, factorial modelling is useful to predict the nutrient requirement of fish for maintenance and growth across the life-cycle under given environmental condition (Shearer, 1995; Lupatsch, 2009).

By considering both, the dose-response model can be useful to determine the dilution effect of nutrient in order to further replacement of one ingredient with another. On the other hand, factorial model is useful to determine the protein and energy requirement on the basis of daily maintenance and growth, which allows fish farmer to develop feeding strategies and to predict fish production. Both models are important on the basis of goal.

6.5. Modelling of nutrient intake and efficiency

The relationship between digestible nutrient intake (protein and energy) and retention was expressed as a linear regression and presented in chapter 5 (Fig. 5.3 and 5.4). The slopes of regression lines which plotted protein gain against digestible protein intake between two temperatures (Fig. 5.3) were significantly different ($F_{1,4}=26.652$, $P=0.007$) and the slope was lower at 19°C indicating that brook trout are less capable of using protein to increase protein gain at elevated temperature. The slopes of regression

lines that modelled energy gain against digestible energy intake at the two temperatures (Fig. 5.4) were not significantly different ($F_{1,4}=2.417$, $P=0.195$) indicating that the relative response of energy retention was the same at both temperatures as the energy intake increased. Considering these findings, it can be concluded that at both temperatures brook trout are equally capable of using dietary non-protein energy sources to achieve growth efficiency.

In chapter 5, factorial modelling mainly focused on protein and energy requirements and nutrient requirements were determined on a nutrient gain basis. Data obtained from experimental chapter 5 were used to model nutrient intake against weight gain to determine the minimum feed, digestible protein and energy requirement for weight gain in this chapter. The maintenance feed requirements for growth of brook trout were $0.79 \text{ g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$ and $0.84 \text{ g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$ at 15°C and 19°C , respectively (Fig. 6.1). The slopes of regression lines of weight gain against feed intake between two temperatures (Fig. 6.1) indicated the amount of food required for per unit of weight gain and can be used to explain the feed efficiency ratio (FER) on a metabolic weight basis. The FER of brook trout was 1.49 and 1.42 at 15°C and 19°C , respectively (Fig. 6.1). The slopes were not significantly different ($F_{1,4}=0.005$, $P=0.947$) indicating that the relative feed efficiency to produce a unit weight gain was the same at both temperatures.

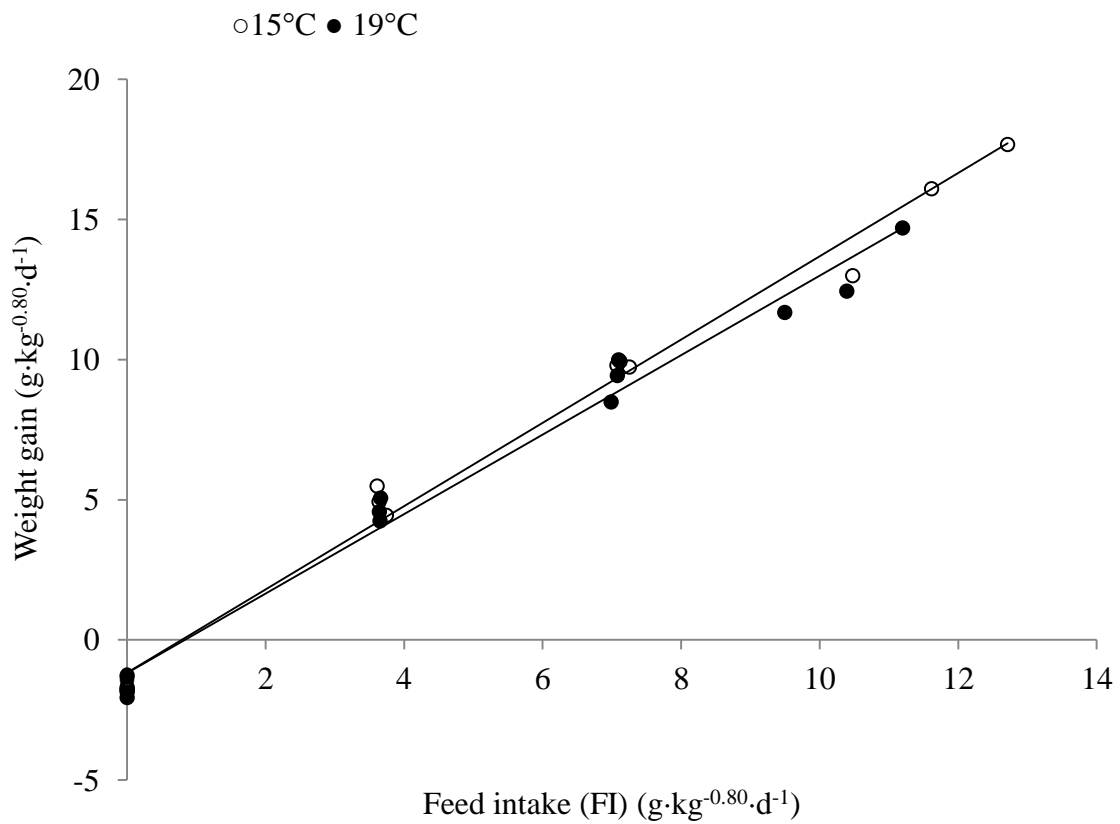


Figure 6.1. The relationship between weight gain ($\text{g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$) and feed intake ($\text{g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$) of brook trout, *Salvelinus fontinalis* at two temperatures, 15°C and 19°C. Weight gain at 15°C = $1.487 \pm 0.050\text{FI} - 1.178 \pm 0.355$ ($R^2=0.99$, $F_{1,10}=874.938$, $P < 0.001$) and weight gain at 19°C = $1.418 \pm 0.058\text{FI} - 1.186 \pm 0.376$ ($R^2=0.99$, $F_{1,10}=607.109$, $P < 0.001$). The maintenance feed requirement for growth of brook trout was $0.79 \text{ g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$ and $0.84 \text{ g}\cdot\text{kg}^{-0.80}\cdot\text{d}^{-1}$ at 15°C and 19°C, respectively.

The maintenance digestible protein requirements for growth of brook trout were $0.22 \text{ g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ at both temperatures (Fig. 6.2). The slopes of regression lines of weight gain against digestible protein intake between two temperatures (Fig. 6.2) indicated that amount of digestible protein required for per unit of weight gain and can be used to explain the protein efficiency ratio (PER) on a metabolic weight basis. The PER of brook trout was 3.69 and 3.62 at 15°C and 19°C , respectively (Fig. 6.2). The slopes were not significantly different ($F_{1,4}=0.006$, $P=0.942$) indicated that the protein efficiency to produce a unit weight gain at both temperatures was similar.

The maintenance digestible energy requirements for growth of brook trout were $14.54 \text{ kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ and $14.64 \text{ kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ at 15°C and 19°C , respectively (Fig. 6.3). The slopes of regression lines of weight gain against digestible energy intake between two temperatures (Fig. 6.3) were not significantly different ($F_{1,4}=0.005$, $P=0.947$) and indicated that the energy efficiency to produce a unit weight gain was the same at both temperatures. In conclusion, although weight gain of brook trout was lower at high temperature, feed and nutrient efficiencies were similar.

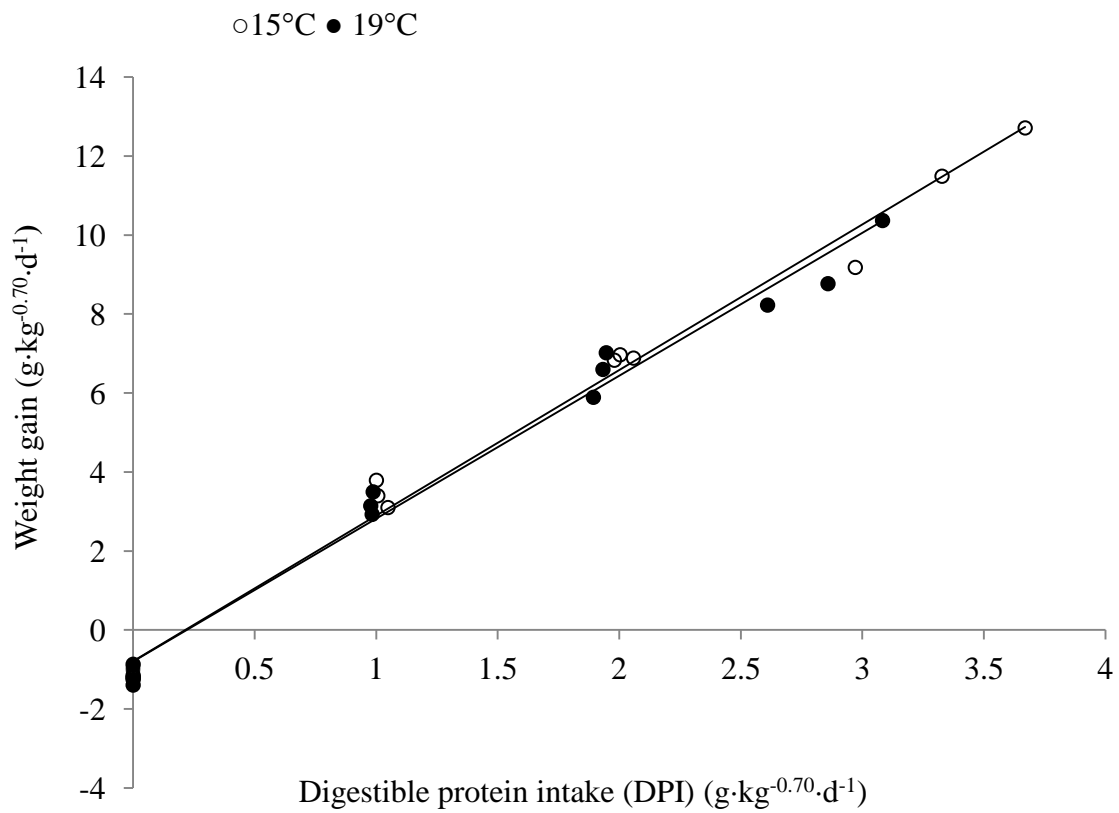


Figure 6.2. The relationship between weight gain ($\text{g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$) and digestible protein intake ($\text{g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$) of brook trout, *Salvelinus fontinalis* at two temperatures, 15°C and 19°C. Weight gain at 15°C = $3.686 \pm 0.121 \text{ DPI} - 0.791 \pm 0.244$ ($R^2=1.00$, $F_{1,10}=921.655$, $P<0.001$) and weight gain at 19°C = $3.616 \pm 0.145 \text{ DPI} - 0.795 \pm 0.259$ ($R^2=1.00$, $F_{1,10}=625.701$, $P<0.001$). The maintenance digestible protein requirement for growth of brook trout was $0.22 \text{ g} \cdot \text{kg}^{-0.70} \cdot \text{d}^{-1}$ at both temperatures.

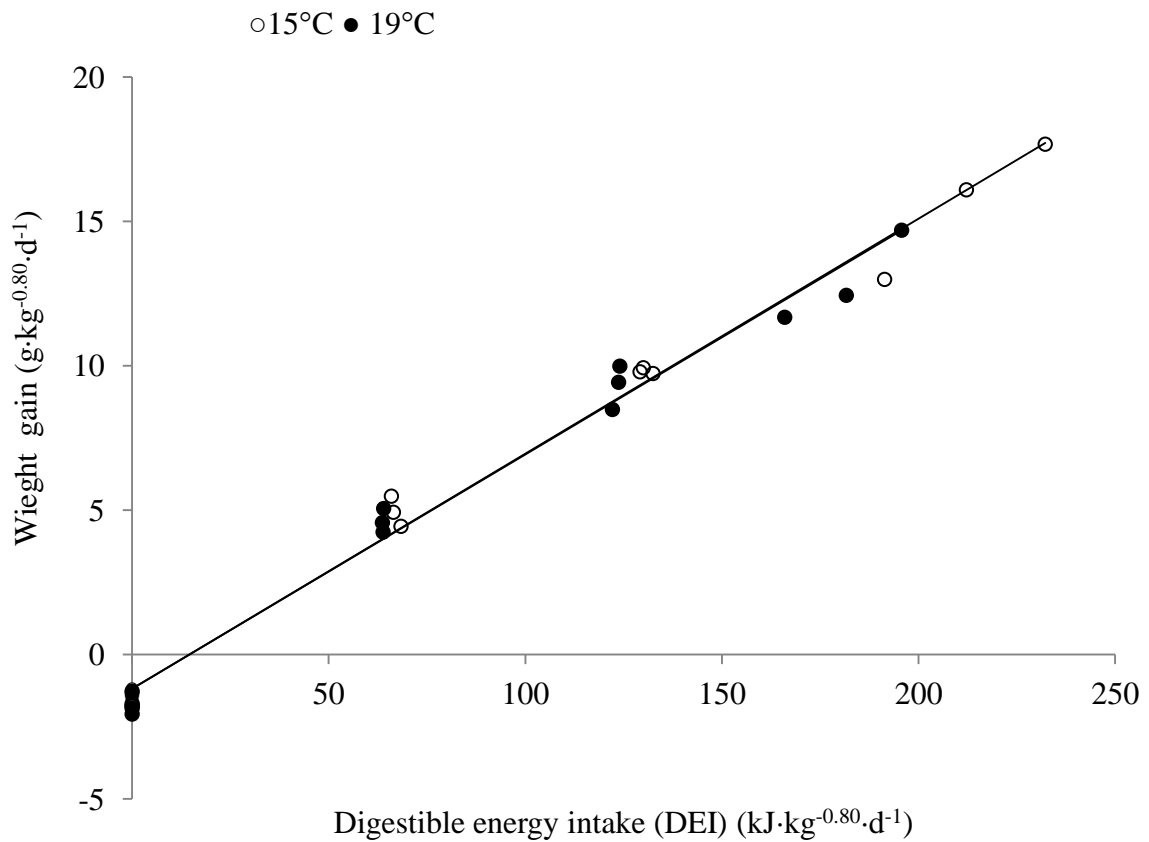


Figure 6.3. The relationship between weight gain ($\text{g} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$) and digestible energy intake (DEI) ($\text{kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$) of brook trout, *Salvelinus fontinalis* at two temperatures, 15°C and 19°C. Weight gain at 15°C = $0.081 \pm 0.003 \text{ DEI} - 1.178 \pm 0.355$ ($R^2 = 0.99$, $F_{1,10} = 874.938$, $P < 0.001$) and weight gain at 19°C = $0.081 \pm 0.003 \text{ DEI} - 1.186 \pm 0.376$ ($R^2 = 0.99$, $F_{1,10} = 607.109$, $P < 0.001$). The maintenance digestible energy requirement for growth of brook trout was $14.54 \text{ kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ and $14.64 \text{ kJ} \cdot \text{kg}^{-0.80} \cdot \text{d}^{-1}$ at 15°C and 19°C, respectively.

6.6. Conclusion

Determination of crude protein requirement of any fish species is necessary prior to any further investigation into alternative protein sources. A major finding from this thesis is the determination of protein requirement of brook trout, can be used as baseline data for further research to replace fish meal based protein with plant protein in the diet of the brook trout. The main protein sources in the diet in all experiments were from fish meal and wheat gluten. It is well accepted that fish meal based diets have balanced amino acid content (Gatlin III et al., 2007). Further research needs to be done to determine the amino acid requirement for brook trout. Brook trout were able to use gelatinised carbohydrate up to 26% level to replace fish oil with no detriment to health, nutrient digestibility or growth. Further investigations are needed to determine the upper threshold of gelatinised carbohydrate inclusion in the diet of brook trout. These findings may be used for developing feeds for other salmonids.

Protein and energy requirement models for brook trout at optimum and elevated summer temperature have been successfully modelled in this thesis (chapter 5). Based on these models a complete diet specification chart has been developed (chapter 5), which will benefit the brook trout farming industry. Implication of efficient feeding management is certainly important to reduce aquaculture waste. Factorial models have been successfully applied to minimise the environmental impact of aquaculture operations (Lupatsch et al., 2003; Hua et al., 2008) and it could be applied for brook trout farming. When applying these feed models to industry it will be important to validate on-farm performance under commercial conditions, where additional metabolic costs through water current and cage dynamics may elevate the maintenance requirement derived in experimental tank system. Feeding strategies obtained from the factorial model is mainly supported by the growth model. For further study, the growth model can be integrated with more holistic way considering specific culture conditions such as salinity, dissolved oxygen especially in sea-cage culture operations where water quality frequently fluctuate with tide and season.

6.5. References

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APPENDICES

APPENDICES

APPENDICES

Appendix A: Weight (mean \pm SD) of brook trout for 28 days starvation

Temp °C	Before starvation		After starvation	
	Mean weight (g·fish ⁻¹) \pm SD	Condition factor, K = (W/L ^b)·1000	Mean weight (g·fish ⁻¹) \pm SD	Condition factor, K = (W/L ^b)·1000
15	5.15 \pm 0.52	8.05 \pm 0.73	4.64 \pm 0.52	7.12 \pm 0.41
	4.97 \pm 0.96	7.98 \pm 0.67	4.33 \pm 0.87	7.02 \pm 1.07
	10.10 \pm 0.60	7.86 \pm 0.48	9.15 \pm 0.57	7.33 \pm 0.33
	10.25 \pm 0.80	8.40 \pm 0.67	9.02 \pm 0.88	7.10 \pm 0.32
	18.08 \pm 2.42	8.01 \pm 2.03	16.80 \pm 2.69	7.34 \pm 0.44
	19.24 \pm 2.12	8.66 \pm 0.59	17.93 \pm 1.98	7.18 \pm 0.38
	196.36 \pm 20.07	7.65 \pm 0.97	186.32 \pm 17.11	6.40 \pm 0.44
	195.70 \pm 16.57	7.39 \pm 0.45	181.13 \pm 14.63	6.21 \pm 0.43
	536.96 \pm 140.45	9.54 \pm 0.84	518.59 \pm 133.27	9.46 \pm 4.60
	567.12 \pm 122.21	10.18 \pm 1.33	545.31 \pm 121.66	8.46 \pm 0.93
19	5.20 \pm 0.82	8.20 \pm 0.65	4.41 \pm 0.85	7.13 \pm 0.36
	4.82 \pm 0.92	7.81 \pm 0.71	4.14 \pm 0.83	6.96 \pm 0.45
	9.86 \pm 0.75	8.20 \pm 0.75	8.76 \pm 0.73	7.06 \pm 0.60
	9.90 \pm 1.01	7.99 \pm 0.45	8.75 \pm 0.93	7.47 \pm 0.40
	15.95 \pm 2.46	8.67 \pm 0.87	14.37 \pm 2.41	7.45 \pm 0.43
	17.24 \pm 2.81	8.76 \pm 0.66	15.55 \pm 2.68	7.78 \pm 0.82
	193.08 \pm 16.54	7.30 \pm 0.68	182.93 \pm 14.44	6.70 \pm 0.53
	187.69 \pm 13.21	7.02 \pm 0.51	177.03 \pm 12.88	6.32 \pm 0.38
	472.20 \pm 88.22	9.15 \pm 0.87	459.64 \pm 86.71	8.30 \pm 0.79
	500.33 \pm 108.33	8.89 \pm 0.53	487.89 \pm 107.70	7.95 \pm 0.57

APPENDICES

Appendix B: Proximate composition of brook trout before and after starvation

Temp °C	Before starvation				After starvation		
	Mean weight (g·fish ⁻¹)	Moisture (%)	Protein (%)	Energy (MJ·kg ⁻¹)	Moisture (%)	Protein (%)	Energy (MJ·kg ⁻¹)
15	5.15	75.04	16.23	6.00	78.84	15.31	4.65
	4.97	75.04	16.23	6.00	78.75	15.35	4.75
	10.10	76.07	15.57	5.77	77.29	15.70	5.35
	10.25	76.07	15.57	5.77	77.32	16.13	5.20
	18.08	73.26	17.41	6.88	74.56	16.85	6.25
	19.24	73.26	17.41	6.88	74.60	16.76	6.49
	196.36	72.42	16.66	7.31	72.80	17.01	6.99
	195.70	72.42	16.66	7.31	72.92	17.37	7.00
	536.96	67.00	17.79	9.11	68.50	17.72	8.68
	567.12	67.00	17.79	9.04	68.95	17.86	8.61
19	5.20	76.38	15.81	5.68	78.70	15.39	4.51
	4.82	76.38	15.81	5.68	79.46	14.78	4.43
	9.86	75.84	15.76	5.82	76.99	16.02	5.25
	9.90	75.84	15.76	5.82	77.36	16.08	5.14
	15.95	73.68	17.14	6.77	74.82	16.64	6.18
	17.24	73.68	17.14	6.77	74.72	16.75	6.22
	193.08	71.78	17.05	7.25	73.61	16.69	6.72
	187.69	71.78	17.05	7.25	73.89	16.70	6.63
	472.20	67.75	17.64	9.23	68.44	17.23	8.57
	500.33	67.75	17.64	9.23	68.13	17.21	8.57

APPENDICES

Appendix C: Protein and energy loss of brook trout for 28 days study

Temp °C	Energy				Protein		
	Mean weight (g·fish ⁻¹)	Initial (kJ·fish ⁻¹)	Final (kJ·fish ⁻¹)	Loss (kJ·fish ⁻¹ ·day ⁻¹)	Initial (g·fish ⁻¹)	Final (g·fish ⁻¹)	Loss (g·fish ⁻¹ ·day ⁻¹)
15	5.15	30.89	21.58	0.33	0.84	0.71	0.004
	4.97	29.84	20.54	0.33	0.81	0.66	0.005
	10.10	58.24	48.90	0.33	1.57	1.44	0.005
	10.25	59.12	46.88	0.44	1.60	1.46	0.005
	18.08	124.33	104.93	0.69	3.15	2.83	0.011
	19.24	132.31	116.42	0.57	3.35	3.01	0.012
	196.36	1435.14	1301.40	4.78	32.71	31.69	0.036
	195.70	1430.31	1268.27	5.79	32.60	31.47	0.040
	536.96	4889.91	4501.62	13.87	95.51	91.88	0.130
	567.12	5127.17	4693.72	15.48	100.88	97.40	0.124
19	5.20	29.55	19.87	0.35	0.82	0.68	0.005
	4.82	27.38	18.34	0.32	0.76	0.61	0.005
	9.86	57.42	45.97	0.41	1.55	1.40	0.005
	9.90	57.65	44.97	0.45	1.56	1.41	0.005
	15.95	107.97	88.76	0.69	2.73	2.39	0.012
	17.24	116.73	96.74	0.71	2.95	2.61	0.012
	193.08	1399.09	1228.66	6.55	32.91	30.53	0.085
	187.69	1360.01	1173.94	6.20	31.99	29.57	0.087
	472.20	4358.49	3938.90	14.99	83.28	79.18	0.146
	500.33	4618.17	4182.53	15.55	88.24	83.99	0.152

APPENDICES

Appendix D: Feed intake, protein and energy retention of brook trout after 28 days of growth trial at 15°C.

	0%	0%	0%	1%	1%	1%	2%	2%	2%	satiation	satiation	satiation
Initial weight (g)	21.55	22.12	21.26	20.98	24.09	21.09	20.63	23.89	21.80	21.69	22.35	23.33
Final weight (g)	19.44	19.85	19.10	28.02	31.08	29.05	35.98	40.93	38.09	44.28	52.71	58.75
Mean weight (g)*	20.47	20.95	20.15	24.24	27.37	24.75	27.25	31.27	28.82	30.99	34.32	37.02
Metabolic body weight kg ^{0.70}	0.066	0.067	0.065	0.074	0.081	0.075	0.080	0.088	0.084	0.088	0.094	0.100
Metabolic body weight kg ^{0.80}	0.045	0.045	0.044	0.051	0.056	0.052	0.056	0.063	0.059	0.062	0.067	0.072
Weight gain (g·fish ⁻¹ ·day ⁻¹)	-0.076	-0.081	-0.077	0.251	0.250	0.285	0.548	0.609	0.582	0.807	1.084	1.265
Weight gain (g·kg ^{-0.70} ·day ⁻¹)	-1.149	-1.217	-1.190	3.398	3.099	3.790	6.830	6.883	6.967	9.179	11.488	12.711
Weight gain (g·kg ^{-0.80} ·day ⁻¹)	-1.695	-1.791	-1.758	4.929	4.441	5.486	9.792	9.733	9.933	12.992	16.094	17.674
Total feed intake (gDM·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	0.186	0.210	0.187	0.396	0.453	0.417	0.650	0.783	0.910
Total feed intake (gDM·kg ^{-0.70} ·day ⁻¹)	0.000	0.000	0.000	2.508	2.612	2.493	4.932	5.128	4.992	7.402	8.291	9.143
Total feed intake (gDM·kg ^{-0.80} ·day ⁻¹)	0.000	0.000	0.000	3.637	3.743	3.609	7.072	7.252	7.117	10.476	11.615	12.713
Digestible protein fed (g·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	0.074	0.084	0.075	0.159	0.182	0.167	0.261	0.314	0.365
Digestible protein fed (g·kg ^{-0.70} ·day ⁻¹)	0.000	0.000	0.000	1.007	1.048	1.001	1.980	2.059	2.004	2.971	3.328	3.670
Digestible energy fed (kJ·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	3.389	3.842	3.419	7.234	8.282	7.614	11.880	14.293	16.619
Digestible energy fed (kJ·kg ^{-0.80} ·day ⁻¹)	0.000	0.000	0.000	66.436	68.367	65.925	129.164	132.461	129.991	191.347	212.152	232.195
Protein gain (g·fish ⁻¹ ·day ⁻¹)	-0.008	-0.008	-0.009	0.037	0.036	0.041	0.079	0.085	0.082	0.101	0.142	0.161
Protein gain (g·kg ^{-0.70} ·day ⁻¹)	-0.108	-0.110	-0.128	0.448	0.405	0.490	0.887	0.869	0.879	1.040	1.360	1.466
Energy gain (kJ·fish ⁻¹ ·day ⁻¹)	-0.976	-1.012	-1.046	1.367	1.417	1.540	3.814	4.141	3.989	5.652	7.847	9.055
Energy gain (kJ·kg ^{-0.80} ·day ⁻¹)	-21.902	-22.295	-23.783	26.788	25.209	29.697	68.096	66.234	68.098	91.038	116.478	126.507

*mean weight (g) = (initial weight x final weight)^{0.5}

APPENDICES

Appendix E: Feed intake, protein and energy retention of brook trout after 28 days of growth trial at 19°C.

	0%	0%	0%	1%	1%	1%	2%	2%	2%	satiation	satiation	satiation
Initial weight (g)	22.58	21.75	20.60	21.56	20.64	21.46	22.19	21.49	20.34	21.68	20.39	21.41
Final weight (g)	20.92	19.19	18.37	27.65	27.04	28.83	38.81	36.62	33.14	41.45	45.61	42.60
Mean weight (g)*	21.73	20.43	19.45	24.41	23.62	24.87	29.34	28.05	25.96	29.98	30.50	30.20
Metabolic body weight kg ^{0.70}	0.069	0.066	0.063	0.074	0.073	0.075	0.085	0.082	0.078	0.086	0.087	0.086
Metabolic body weight kg ^{0.80}	0.047	0.044	0.043	0.051	0.050	0.052	0.059	0.057	0.054	0.060	0.061	0.061
Weight gain (g·fish ⁻¹ ·day ⁻¹)	-0.060	-0.092	-0.080	0.218	0.228	0.263	0.594	0.541	0.457	0.706	0.901	0.757
Weight gain (g·kg ^{-0.70} ·day ⁻¹)	-0.868	-1.395	-1.255	2.926	3.142	3.496	7.018	6.596	5.891	8.225	10.366	8.767
Weight gain (g·kg ^{-0.80} ·day ⁻¹)	-1.273	-2.059	-1.861	4.242	4.570	5.058	9.988	9.429	8.487	11.681	14.695	12.441
Total feed intake (gDM·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	0.187	0.182	0.191	0.422	0.406	0.377	0.574	0.686	0.632
Total feed intake (gDM·kg ^{-0.70} ·day ⁻¹)	0.000	0.000	0.000	2.519	2.504	2.529	4.986	4.951	4.851	6.686	7.898	7.323
Total feed intake (gDM·kg ^{-0.80} ·day ⁻¹)	0.000	0.000	0.000	3.651	3.641	3.660	7.096	7.078	6.988	9.495	11.196	10.391
Digestible protein fed (g·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	0.073	0.071	0.074	0.165	0.158	0.147	0.224	0.268	0.247
Digestible protein fed (g·kg ^{-0.70} ·day ⁻¹)	0.000	0.000	0.000	0.983	0.977	0.987	1.946	1.933	1.894	2.610	3.083	2.859
Digestible energy fed (kJ·fish ⁻¹ ·day ⁻¹)	0.000	0.000	0.000	3.273	3.180	3.330	7.370	7.091	6.581	10.032	11.994	11.046
Digestible energy fed (kJ·kg ^{-0.80} ·day ⁻¹)	0.000	0.000	0.000	63.810	63.643	63.963	124.017	123.701	122.137	165.949	195.682	181.611
Protein gain (g·fish ⁻¹ ·day ⁻¹)	-0.013	-0.016	-0.014	0.033	0.034	0.044	0.087	0.080	0.065	0.092	0.121	0.102
Protein gain (g·kg ^{-0.70} ·day ⁻¹)	-0.218	-0.278	-0.259	0.517	0.543	0.680	1.183	1.126	0.966	1.236	1.597	1.366
Energy gain (kJ·fish ⁻¹ ·day ⁻¹)	-1.040	-1.172	-1.119	0.918	0.943	1.159	3.477	3.314	2.674	4.280	6.043	5.081
Energy gain (kJ·kg ^{-0.80} ·day ⁻¹)	-22.252	-26.343	-26.153	17.890	18.881	22.261	58.504	57.802	49.622	70.805	98.586	83.533

*mean weight (g) = (initial weight x final weight)^{0.5}

APPENDICES

Appendix F: Commercial growth data of brook trout collected from Tasmania

Mean weight (g)	Temperature °C	Weight gain (g·fish ⁻¹ ·day ⁻¹)	Mean weight (g)	Temperature °C	Weight gain (g·fish ⁻¹ ·day ⁻¹)
0.08	6.1	0.003	11.20	16.6	0.400
0.08	6.1	0.004	13.20	14.7	0.550
0.10	8.0	0.007	14.00	16.7	0.943
0.11	8.9	0.010	15.30	16.0	0.640
0.11	8.0	0.005	17.60	16.0	0.440
0.14	8.0	0.010	18.50	16.9	0.625
0.14	8.0	0.007	20.40	16.8	0.786
0.18	6.6	0.034	20.60	15.7	0.600
0.19	9.1	0.005	23.50	16.8	0.329
0.20	6.6	0.007	25.40	16.5	0.514
0.22	9.6	0.017	25.80	16.1	0.613
0.25	9.2	0.018	25.90	16.1	0.607
0.39	11.7	0.037	29.00	16.5	0.923
0.42	9.2	0.021	35.00	15.6	2.833
0.47	9.6	0.037	35.00	15.6	1.833
0.59	11.7	0.027	41.00	15.8	1.857
0.65	12.8	0.079	44.00	16.1	0.429
0.69	12.2	0.053	52.00	15.8	0.857
0.78	12.8	0.031	54.00	15.8	1.143
1.00	10.2	0.093	58.00	16.1	2.571
1.11	12.6	0.100	62.00	15.7	1.000
1.20	10.2	0.096	75.00	17.3	4.000
1.65	13.5	0.141	76.00	17.1	2.857
1.71	14.4	0.099	92.00	18.5	0.571
1.87	13.5	0.221	96.00	18.5	0.286
2.40	13.5	0.123	96.00	17.4	1.000
2.64	11.4	0.066	98.00	17.4	5.833
3.10	13.1	0.154	102.00	16.0	4.125
3.26	13.0	0.180	111.00	15.1	2.625
3.60	13.1	0.221	132.00	14.0	1.833
4.18	13.0	0.183	133.00	16.0	2.125
4.52	14.0	0.226	135.00	14.4	1.833
5.15	13.0	0.186	143.00	13.8	5.875
5.46	12.1	0.307	146.00	12.5	2.889
6.10	13.4	0.400	150.00	14.4	1.833
6.45	12.1	0.192	161.00	12.5	3.861
7.30	13.7	0.275	190.00	13.7	5.857
7.60	13.7	0.288	300.00	10.0	7.143
8.90	14.4	0.329	320.00	10.9	2.143
9.50	14.9	0.186	350.00	10.1	2.727
9.90	14.9	0.471	350.00	9.2	2.308
10.80	14.7	0.563	380.00	7.4	7.833